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THE FINE STRUCTURE OF HUMAN ATHEROSCLEROTIC LESIONS

JACK C. GEER, M.D.*; HENRY C. MCGILL, JR., M.D., AND JACK P. STRONG, M.D.*

From the Department of Pathology, Louisiana State University School of Medicine, New Orleans, La.

Despite widespread application of electron microscopy to the study of a variety of tissues, few reports have appeared concerning the fine structure of normal muscular or elastic arteries [1-3] or arterial lesions.[4-7] This scarcity is understandable in view of the difficulties we have experienced obtaining good preservation of fine structure in large arteries. Conventional methods of fixation and embedding for electron microscopy applied to large arteries have usually resulted in severe distortion of cellular and interstitial elements. Arterial injection of osmium fixative provided excellent preservation of muscular arteries in the rat. We have found that human arterial tissue obtained at necropsy, fixed in formalin and postfixed in osmium showed good preservation. This report describes briefly the fine structure of normal arteries and, in more detail, human atherosclerotic lesions, particularly fatty streaks, in aortas and coronary arteries.

MATERIAL AND METHODS

Aortas and mesenteric arteries were obtained from rats and dogs; human femoral and renal arteries were procured from amputation specimens and resected kidneys. Small portions of artery were flooded with buffered osmium fixative [8] with added sucrose [9] immediately after removal from the anesthetized animal or patient. Blocks measuring 1 by 1 by 4 mm. were cut from the artery and immersed in osmium fixative for one hour. Rat mesenteric artery was prepared by arterial injection of

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* Senior Research Fellows, United States Public Health Service.

osmium fixative. The peritoneal cavity was opened under ether anesthesia and the aorta ligated at its bifurcation; the osmium fixative was injected by syringe and needle into the abdominal aorta under moderate pressure until the small vessels over the intestine were blackened. The artery was then dissected from the mesentery, cut into 5 mm. segments, and fixed in osmium for an additional hour.

Human coronary arteries and aortas were obtained from 13 necropsies (Table I). The aorta was opened longitudinally and placed in neutral buffered[10] 10 per cent formalin. The coronary arteries were dissected from the heart, partially sectioned transversely at 1 to 2 cm. intervals, and placed in buffered formalin. After 12 to 24 hours in formalin the arteries were freed of adventitial fat and opened longitudinally. Blocks measuring 1 by 1 by 4 mm. were cut from grossly normal and abnormal intima of coronary arteries and aortas. Each block was identified for anatomic location and gross appearance—that is, whether the intima was normal or the seat of fatty streak, fibrous plaque, thrombosed plaque, or hemorrhagic plaque. Blocks were fixed in buffered osmium for one hour, dehydrated in graded methyl alcohols, and embedded in a 1:3 mixture of methyl and butyl methacrylates.

TABLE I

HUMAN ARTERIES OBTAINED FOR ELECTRON MICROSCOPY

Case no.	Age (yr.)	Sex	Race	Hr. post-mortem	Necropsy diagnosis	Examined by electron miscroscpoy	
						Aorta	Coronaries
1	7	F	W	1	Diabetes mellitus (2 yr.); cerebral edema	+	—
2	16	F	N	4	Cerebellar astrocytoma	+	+
3	18	F	N	4	Brain abscess	+	+
4	22	F	W	3	Pulmonary embolus	+	+
5	23	M	W	2	Subarachnoid hemorrhage; berry aneurysm; coarctation of aorta	—	+
6	25	M	N	4	Bronchogenic carcinoma	+	+
7	26	F	W	2	Asthma, bronchopneumonia	+	+
8	28	M	W	1	Hemorrhagic pancreatitis	+	+
9	29	M	N	3	Epilepsy; acute renal failure	+	+
10	31	F	W	4	Monocytic leukemia	+	+
11	35	M	W	1	Bronchogenic carcinoma	+	+
12	38	M	W	1	Duodenal ulcer; massive gastrointestinal hemorrhage	+	—
13	56	M	W	2	Myocardial infarction	+	+

Two μ sections cut from the plastic-embedded tissue were mounted on albumin-coated glass slides. The embedding plastic was removed with xylol, and the osmium, by flooding the slide with 3 per cent hydrogen peroxide for 5 minutes. These sections were stained with Weigert's resorcin-fuchsin for 12 hours; hematoxylin, 2 minutes; and van Gieson's stain, 3 minutes. Such sections served as guides for trimming the plastic blocks for thin sectioning, and for comparison with the light microscope appearance of carbowax-embedded portions of the lesions. Thin sections for electron microscopy were cut on a Servall Porter-Blum microtome, mounted on formvar coated copper grids, and viewed and photographed in an RCA EMU-3 electron microscope.

After removing blocks from the aorta or coronary arteries for osmium fixation, additional blocks were cut from adjacent areas for embedding in carbowax. Serial sections of these were cut and stained with oil red O, Weigert-van Gieson, periodic acid-Schiff-Alcian blue,[11] and hematoxylin and eosin.

Results

Normal Arterial Structure

In general, preservation of arterial wall elements in both human and animal specimens was poor when the fresh tissue was fixed immediately in osmium. The internal elastic membrane was folded, and elastic fibers often appeared swollen and of decreased density. Endothelial cells were polypoid in configuration, and large vacuoles were present in smooth muscle and interstitial tissue. It was concluded that these changes resulted from contraction of the vessel and stress on the fixed tissue during polymerization of the plastic.

The best preservation of arterial fine structure was obtained in the mesenteric arteries of rats injected with osmium fixative. Endothelial cells in these arteries had a centrally placed nucleus causing the cell to bulge slightly into the lumen (Fig. 1). Structures within endothelial cells were similar in rats and dogs and conformed to previous descriptions.[12–19] Endothelial cell cytoplasm contained vesicles measuring 40 to 60 mμ in diameter. These were most numerous along the cytoplasmic membrane with which they often fused. There were also mitochondria, endoplasmic reticulum, clear vacuoles measuring up to 0.5 μ, and a small Golgi zone adjacent to the nucleus. Occasional dense bodies, similar to those described by Buck,[16,18] were found. Some endothelial cells had slender cytoplasmic processes projecting into the vessel lumen (Fig. 4). The cytoplasmic membrane showed numerous areas of discontinuity interpreted as artifacts of fixation. The endothelial cell was directly apposed to the internal elastic membrane or was separated from it by unit fibers of collagen (Fig. 1).

Elastic tissue was homogeneous and moderately dense (Fig. 1). Only rarely was fibrillar material evident in the elastic fibers.[3] There were numerous openings in the internal elastic membrane, through which extended unit fibers of collagen (Fig. 1). Portions of endothelial cytoplasm also often extended through membrane in contracted arteries, but rarely in pressure-fixed arteries.

The muscular artery had a prominent internal elastic membrane, and there were collagen and small elastic fibers between smooth muscle cells of the media (Fig. 1). The thoracic aorta had no single internal elastic membrane; instead, there were many small elastic fibers and smooth muscle. The abdominal aorta had an internal elastic membrane similar to that of muscular arteries. The media of elastic arteries was composed

of closely applied large elastic fibers and smooth muscle with intervening spaces containing collagen fibers.

The smooth muscle cell in the media of arteries fixed under pressure had a fusiform shape and a central elongated nucleus (Fig. 1). Smooth muscle cells in vessels fixed after removal from the body were irregular in shape, being generally shorter and broader. Mitochondria and endoplasmic reticulum lay at the extremities of the nucleus in a portion of cytoplasm devoid of myofilaments (Fig. 1). A small Golgi zone was adjacent to the nucleus. Myofilaments were arranged in the long axis of the cell, and varied in thickness from 10 to 30 mμ. Among the myofilaments were focal fusiform areas of density (Figs. 1 and 2). Along the cytoplasmic membrane were vesicles measuring from 40 to 80 mμ in diameter that approached and often fused with the cytoplasmic membrane (Fig. 2). Descriptions of smooth muscle have been given for blood vessels [3,7,15]; ureter [20]; uterus [21–23]; urinary bladder [21]; and gallbladder [21]; the findings in this study are essentially the same. An additional characteristic feature of smooth muscle in arteries was the dense area lying along the cytoplasmic membrane; this was usually triangular in shape with the base of the triangle on the cytoplasmic membrane and the apex directed in the long axis of the myofilaments. Myofilaments appeared to enter these triangular densities (Fig. 2). Smooth muscle cells in muscular arteries were surrounded by a basement membrane 30 to 80 mμ in thickness (Fig. 2). Between smooth muscle cells in the media of the rat mesenteric artery were unit fibers of collagen and small elastic fibers (Figs. 1 and 2). Smooth muscle cells in the aortic media of both animals and man seldom had a basement membrane; they were applied to elastic plates often with interposed unit fibers of collagen.

The only cells in the intima and media of muscular arteries were smooth muscle and endothelium. In the adventitia of muscular arteries there were fusiform cells among the collagen fibers, presumably fibrocytes. Rarely in the media of the dog aorta there were cells that did not appear to be smooth muscle, but they did not resemble adventitial fibrocytes.

The smooth muscle cells and connective tissue elements in human renal and femoral arteries were similar to those in animal arteries.

Human Formalin-Osmium Fixed Arteries

The aortas and coronary arteries had grossly visible atherosclerotic lesions of average degree for their age, sex, and race group with two exceptions—cases 1 and 7. Case 1, a 7-year-old white diabetic girl, had more than an average number of fatty streaks and a fibrous plaque in one iliac artery, findings that correspond to the widely recognized pre-

disposition of diabetic individuals to accelerated atherogenesis. Case 7, a 26-year-old white asthmatic woman, had a fibrous plaque in the anterior descending branch of the left coronary artery, producing 75 per cent occlusion of the lumen, distinctly more than average for her age and sex group.

Osmium-fixed human arterial tissue obtained 30 minutes to 1 hour after death without prior formalin fixation was poorly preserved. Fine structure in formalin and osmium-fixed human vessels obtained at necropsy was not as well preserved as in optimally fixed animal artery; the principal alteration attributable to autolysis or formalin was clumping of nuclear chromatin, and swelling and disruption of mitochondria (Fig. 3).

The grossly normal intima of human aortas and coronary arteries varied in thickness and with light microscopy consisted of endothelium, "cells" (smooth muscle and unidentified cells), collagen, and elastic fibers. Electron micrographs confirmed the presence of collagen and elastic fibers, and disclosed most of the cells to be smooth muscle. Some cells could not be definitely identified as smooth muscle by the presence of myofilaments though other features of smooth muscle were present. Only rarely were histiocytes or plasma cells found (Fig. 4). The musculo-elastic intima differed from the intima of animal arteries; the latter consisted only of endothelium, a small amount of collagen, and an internal elastic membrane or lamina.

Human aortic and coronary endothelial cells were similar to those in experimental animals, except they contained more endoplasmic reticulum, principally rough-profiled; and frequently contained dense cytoplasmic inclusions (Figs. 4 and 5). The dense inclusions varied from 0.3 to 2.5 μ in diameter and had no limiting membrane. The most common form consisted of an outer zone of homogeneous, moderately dense material with a core of a more dense homogeneous substance (Fig. 5). The dense core usually showed knife striations (Fig. 5), and was often shattered or dropped out of the section (Fig. 4), suggesting that it was composed of a hard material. These inclusions occurred in endothelial cells in both "normal" and abnormal intima.

Smooth muscle cells in human vessels were similar to those in experimental animals except for nuclear and mitochondrial alterations attributable to autolysis or formalin (Fig. 3). Collagen and elastic fibers were as well preserved as in optimally fixed animal arteries.

Aortic and coronary fatty streaks were characterized grossly as flat or slightly elevated yellow streaks on the intimal surface. Fatty streaks from young individuals, showing little deviation from normal structure by light microscopy, were presumed to represent earlier lesions than

those containing abundant foam cells and increased amounts of collagen. Fibrous plaques were elevated gray-white intimal lesions which on cross section had a yellow grumose core covered by a fibrous cap. The lipid content of both types of lesions was established by the presence of oil red O-stained material in the carbowax sections.

In the electron micrographs of all types of atherosclerotic lesions, most of the identifiable lipid was intracellular and occurred in 3 forms. The first was a reticulated cytoplasmic inclusion containing strands of dense material varying from 3 to 80 mμ in thickness (Figs. 6 to 8). Such inclusions measured up to 1.5 μ in diameter, were limited by a double membrane, and often contained a central dense area from which strands radiated (Figs. 6 and 8). The limiting membrane measured 15 mμ in thickness, and was composed of two 5 mμ membranes separated by a 5 mμ clear space (Fig. 8). In some inclusions, a central dense area occupied nearly the entire inclusion with only a narrow margin of filamentous material; in others, there was an eccentric clear oval area with one border on the limiting membrane (Fig. 6). These reticulated inclusions were found most often in smooth muscle, but were present occasionally in foam cells. They did not arise in endoplasmic reticulum, nor did they represent an alteration of mitochondria (Fig. 7). The second type of lipid appeared as dense, homogeneous cytoplasmic inclusions with no limiting membranes, often with ovoid clear areas at one margin (Fig. 9). This form of inclusion was found principally in smooth muscle cells, and occasionally in foam cells. The third type of lipid appeared as clear vacuoles in foam cells for the most part, but in smooth muscle as well (Figs. 10 and 11).

The only extracellular structures definitely identified as lipid were clear spaces with rectangular or rhomboid configuration, representing cholesterol clefts (Fig. 12). Similar but smaller clefts were observed rarely in foam cells. The nature of the crystalline deposit in the intracellular clefts is unknown; they may have represented fatty acid crystals produced by cooling the tissue below body temperature in the process of fixation. Two other interstitial structures could represent lipid. One was a whorl of laminated dense membranes measuring up to 1.5 μ; this also appeared in foam cells (Figs. 13 and 14). Because of a superficial resemblance to myelin, these are referred to as "myelin forms." The other structure, never observed in cells, was a round or ovoid dense body measuring up to 0.75 μ. This was either homogeneous or had a clear area in its center (Figs. 9 and 13). These two forms occurred only in lesions containing abundant intracellular lipid; therefore, study of contiguous carbowax-embedded sections stained with oil red O could neither confirm nor eliminate a lipid content.

The foam cell, usually occurring in the advanced fatty streak and fibrous plaque, was composed principally of clear vacuoles with little other recognizable cytoplasmic content. The nucleus was indented as though compressed by the lipid vacuoles (Fig. 10). In addition to "myelin forms" and the clefts described above, these cells also occasionally contained moderately dense, homogeneous inclusions which probably corresponded to PAS-positive droplets noted in carbowax sections (Fig. 15).

The form of the lipid found in atherosclerotic lesions was correlated with the type of lesion—small or early fatty streak, advanced fatty streak or fibrous plaque. In the early fatty streak the lipid lay almost exclusively in smooth muscle; it was either reticulated or of the clear vacuolar type, more often the former. Lipid in the more advanced fatty streak often appeared in smooth muscle and foam cells, but was also observed in unidentifiable cells. In advanced fatty streaks the lipid was mostly of the clear vacuolar and dense homogeneous types. Reticulated lipid was present but to a lesser extent than in early fatty streaks. Smooth muscle cells often contained so many vacuoles that they appeared to be in a transitional stage between smooth muscle and foam cells (Fig. 11).

In some advanced fatty streaks there were unidentifiable lipid-containing cells with a granular electron-dense cytoplasm (Fig. 13). Nuclei in these cells were small and dense. Since nuclear preservation was generally poor, however, no conclusions could be based on their appearance. The cells were most numerous in lesions containing many extracellular myelin forms and small dense bodies. The alteration probably reflected cellular degeneration rather than autolysis or a fixation artifact since adjacent cells were well preserved.

Lesions identified grossly as fibrous plaques contained the same elements as fatty streaks, but in different proportions. The fibrous plaque contained much more collagen, with a cap of collagen overlying lipid-containing cells in the intima. The collagenous cap was composed of unit fibers of collagen, unidentified fusiform cells (possibly fibrocytes) occasionally containing homogeneous dense cytoplasmic inclusions, small elastic fibers and numerous extracellular bodies identical to the small dense bodies in fatty streaks. In the lipid core of the fibrous plaque, intracellular lipid, extracellular "myelin forms," and small dense bodies were abundant.

No substance identifiable as fibrin was found in any of the lesions except in a coronary artery of case 12; this was a large fibrous plaque into which there had been hemorrhage.

Discussion

Electron microscopic observations on atherosclerotic lesions must be oriented with respect to the natural history of the process as well as the gross and histologic characteristics. In a disease where lesions develop over a period of many years, vary with sex and race, and show marked individual variation within a given age, sex, racial or ethnic group, it is not expected that observations on a few isolated cases will tell the entire story. Emphasis in this study has been focused on what we believe are the earliest distinctive alterations of atherosclerosis—lipid deposits in the arterial intima. The changes that precede lipid accumulation, the origin and nature of the lipid, and the fate of the fatty streak are the principal questions to which this investigation is addressed.

The fibromuscular intimal layer in the human aorta and coronary artery has been described by others.[24–29] Whether this layer represents the early lesion of atherosclerosis or is a normal developmental phenomenon predisposing to atherogenesis cannot be determined from the limited number of cases examined. The term "atherosclerosis" is used in this paper to denote an arterial intimal lesion in which fat is present.

Two facts concerning the lipid in human fatty streaks that have been established are the variation in its structure and its predominantly intracellular localization, particularly in smooth muscle. The possibility that the different forms of lipid in the lesions result from autolysis must be considered. This is highly unlikely, however, since the occurrence of the different patterns was not related to the length of the postmortem period. In some cases, indeed, all forms could be found in a single tissue section. Moreover, morphologic variations in the reticulated type of lipid inclusion (Figs. 6 to 8) were not correlated with time elapsing between death and necropsy. In those cases where preservation of this type of inclusion appeared best, the postmortem interval ranged from 1 to 4 hours.

The reticulated, dense homogeneous and clear vacuolar lipid inclusions are probably morphologic reflections of chemical differences in the lipid component. Osmium reacts readily with unsaturated lipids and in effect denatures them so that they are no longer soluble in lipid solvents.[30–31] Thus, droplets composed of unsaturated lipid should appear as dense bodies in electron microscopy because of osmium deposition. Droplets of saturated lipid should be represented as clear vacuoles, since osmium is not deposited upon it and it remains soluble in lipid solvents and is thus dissolved away in the processing of the tissue. Observations on optimally fixed animal tissue have shown variations in lipid structure similar to those in arteries obtained at necropsy and fixed in formalin

and osmium.[16,32] This observation strengthens the conclusion that the variations found were not due to autolysis or to formalin fixation. Reticulated lipid inclusions similar to those in human arteries occur in the brown fat of rodents.[33–36] In this type of "structured" inclusion it is possible that the dense strands represent the protein portion of a lipoprotein or the unsaturated portion of an oriented lipid, while the clear areas are the sites once occupied by saturated lipid. Studies designed to correlate the chemical composition of various lesions with structure are needed to determine the nature of the various lipid inclusions.

A second observation, indicating a predominantly intracellular localization of lipid, is firmly established by this study. Furthermore, most of the lipid-containing elements, especially those in early fatty streaks, are smooth muscle. Smooth muscle cells containing vacuoles, presumably of lipid nature, have been observed previously by light microscopy in human and experimental atherosclerotic lesions.[37] Parker's electron microscopic study[7] of experimental coronary atherosclerosis depicted vacuoles in smooth muscle. These were interpreted to "represent sites of lipid." Our observations show that practically all of the lipid is in smooth muscle in early human fatty streaks. The problem, therefore, is the determination of the mechanism by which the smooth muscle cell accumulates lipid. We are unaware of the capacity for phagocytosis by smooth muscle, and it is difficult to believe that initial accumulation occurs by phagocytosis of lipid filtered from the blood. A more likely possibility is the synthesis of lipid by the smooth muscle cell itself. The arterial wall has the capacity of synthesizing various lipids.[38–43] Since the smooth muscle cell is the predominant component of the arterial wall, it is reasonable to presume that lipid synthesis is a function of this cell. Defining the factors, local and systemic, that govern this synthesis is a problem for future research.

Our observation of transitional stages between lipid-containing smooth muscle and foam cells supports Altschul's suggestion[37] that foam cells may arise from smooth muscle. In addition to accumulating abnormal lipid under certain conditions, smooth muscle in the normal arterial wall may have other functions than furnishing contractile protein. The virtual absence of cells resembling fibrocytes in the vascular intima and media raises questions as to the cell responsible for the formation and maintenance of elastic and collagen fibers. This activity could be a function of the smooth muscle cell in this location.

The dense inclusions in the cytoplasm of endothelial cells overlying "normal" intima and initial lesions are probably of lipid nature or are, at least partly, lipid. Lipid droplets were observed in the endothelium in oil red O-stained carbowax sections; the dense inclusions were the

only possible corresponding structures in electron micrographs. It is unlikely that the inclusions represent lipid being transported through the endothelium into the intima. None were observed being extruded into the intima, and similar inclusions were never found in the intima.

The existence of lipid-containing cells with cytoplasmic changes suggesting degeneration provides a possible explanation for the source of extracellular lipid. The latter may, in turn, serve as a stimulus for reactive fibrosis and the progression of lesions to more advanced stages. Substantiation of this hypothesis is impossible until methods can be developed for the positive identification of extracellular lipid. Since fatty streaks and fibrous plaques differed only in the proportions of the various elements present—increased collagen in fibrous plaques—it is probable that fatty streaks are the precursors of fibrous plaques.

Summary

Electron microscopy of elastic and muscular arteries in the normal dog and rat disclosed the fine structure of cellular and connective tissue elements to be similar to that described for endothelium, smooth muscle, elastic fibers and collagen fibers in other organs.

Human arterial tissue procured at necropsy, fixed in buffered formalin and postfixed in osmium tetroxide was, with certain limitations, satisfactory for electron microscopy. This method was used in the study of human atherosclerotic lesions with particular emphasis on early fatty streaks in the coronary arteries and the aorta.

In young individuals the lipid in small intimal fatty streaks lay predominantly in smooth muscle cells and had a reticulated structure. In more advanced streaks from older individuals and in fibrous plaques, intracellular lipid appeared either as clear vacuoles or dense homogeneous inclusions. The lipid-containing cells in more advanced lesions, though occasionally identified as smooth muscle, were often not identifiable. Some smooth muscle elements contained such large numbers of lipid inclusions that they appeared to be in transition to foam cells.

The existence of cytoplasmic lipid inclusions in smooth muscle was interpreted as evidence of lipid synthesis *in situ*. The initial lipid deposition in human atherosclerosis could, therefore, be due to an alteration in the smooth muscle metabolism, causing intracellular lipid to accumulate in abnormal amounts. The different forms of lipid in the various lesions were believed to be due to chemical differences, principally degrees of unsaturation.

In the more advanced stages of human atherosclerosis there were structures in the interstitial tissue thought to represent lipid and other forms representing cholesterol clefts. There were also cells with degen-

erative cytoplasmic changes. The hypothesis is proposed that degeneration of lipid-containing cells may lead to the extravasation of lipid particles into the extracellular spaces. This, in turn, may serve as the stimulus to fibrosis and the progression of lesions to more advanced stages.

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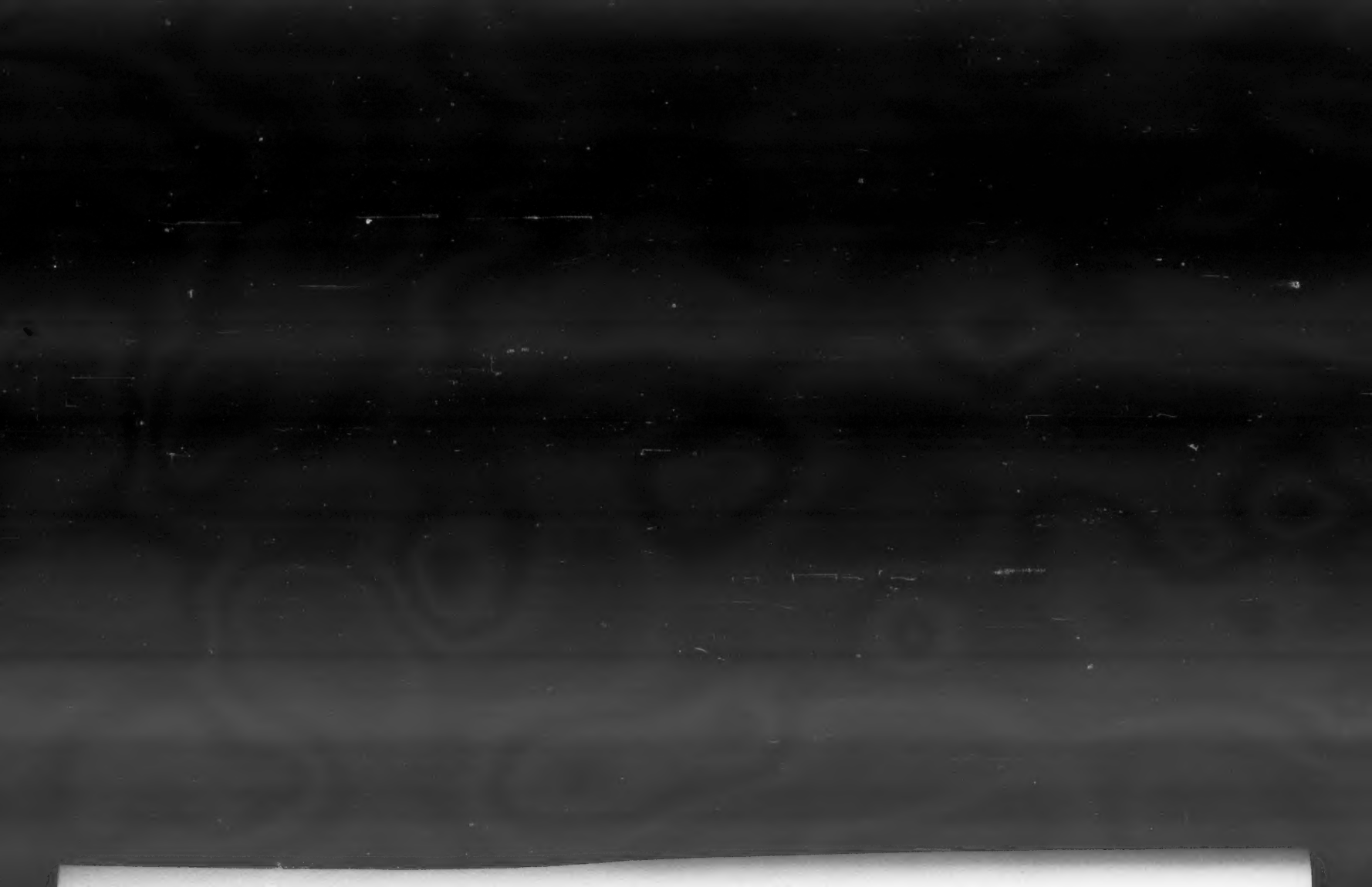
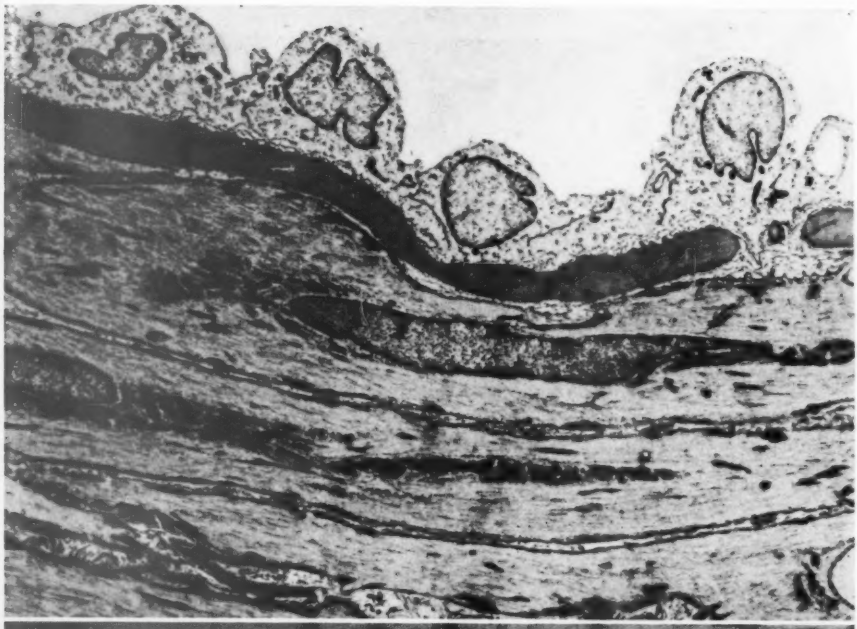
[*Illustrations follow*]

Legends for Figures

FIG. 1. Rat, mesenteric artery. Endothelial cells are flattened along the internal elastic membrane, and are either applied directly to the membrane or separated from it by unit fibers of collagen. The elastic tissue is moderately dense and homogeneous. There is a gap in the internal elastic membrane containing unit fibers of collagen. Smooth muscle cells are fusiform and contain prominent myofilaments. Focally there is increased density in the myofilaments and in the cytoplasmic membrane of the smooth muscle cells. Between the smooth muscle cells are collagen and elastic fibers. Osmium fixation under moderate pressure. × 5,250.

FIG. 2. Rat, mesenteric artery. The cytoplasm of smooth muscle cells contains triangular areas of density along the cytoplasmic membrane. Myofilaments appear to enter these areas. Osmium fixation under moderate pressure. × 34,500.





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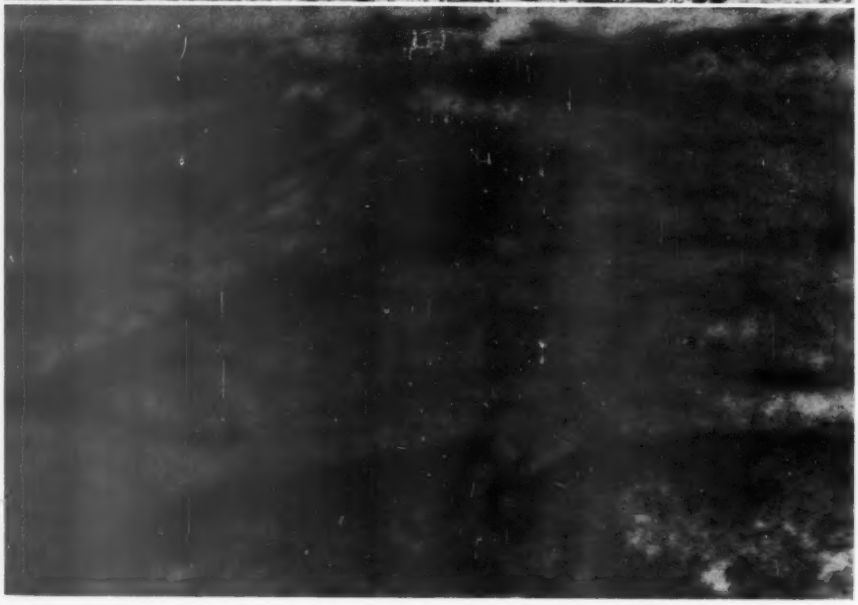

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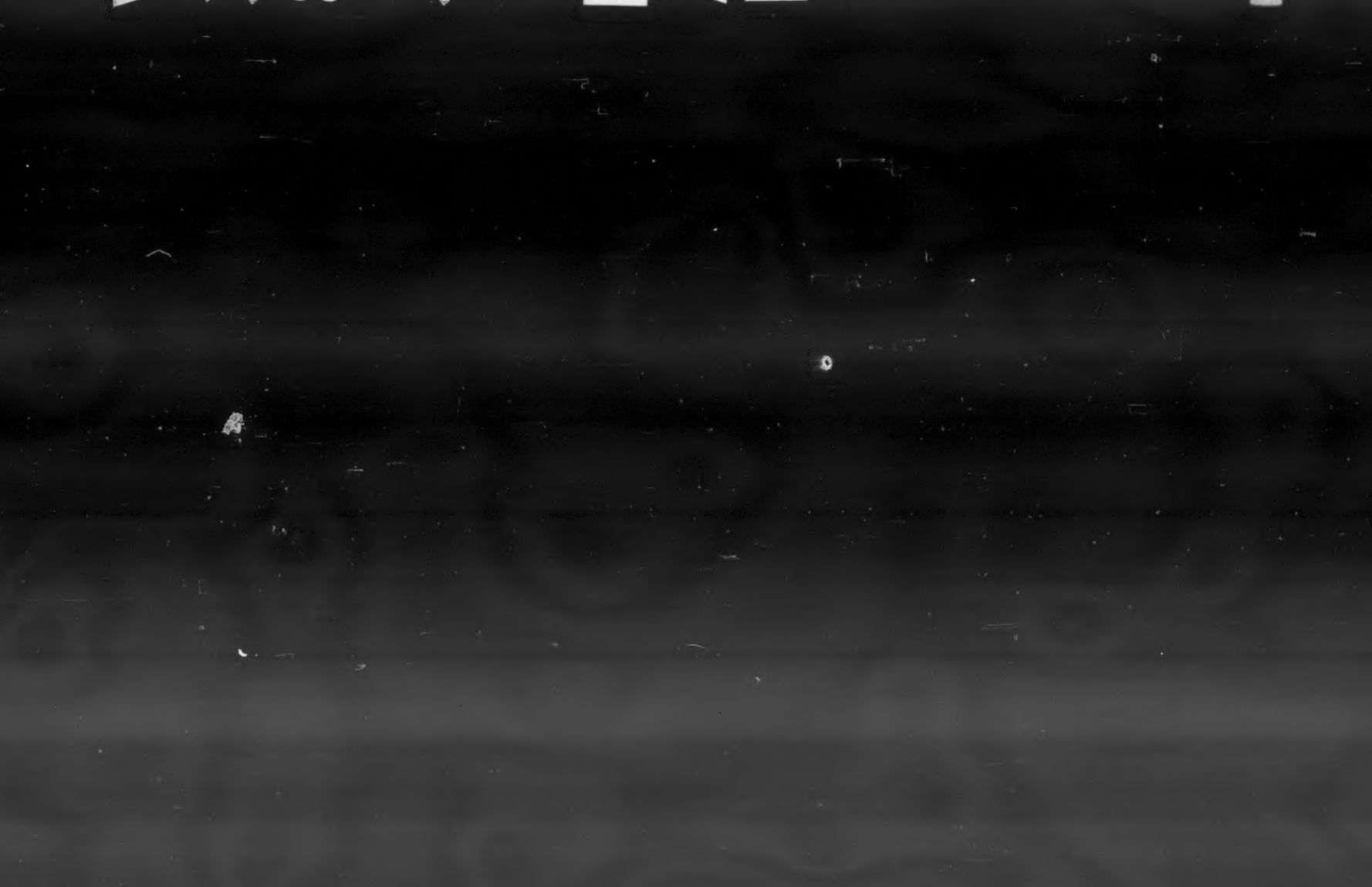
FIG. 3. Human coronary artery, case 4. Smooth muscle cell. The chromatin is clumped at the nuclear membrane. Mitochondria are swollen (arrow). Myofilaments are well preserved. Formalin-osmium fixation, 2 hours post mortem. × 12,600.

FIG. 4. Human coronary artery, case 3. Endothelium and subendothelial plasma cell. The endothelial cell has numerous small projections of cytoplasm into the lumen. The cytoplasm contains an abundance of rough-profiled endoplasmic reticulum and a large, very dense inclusion. The central portion of the latter appears to have shattered and fallen away during sectioning. The abundant endoplasmic reticulum in the subendothelial cell and the paranuclear zone containing mitochondria are characteristic of plasma cells. Formalin-osmium fixation, 4 hours post mortem. × 11,000.

FIG. 5. Human thoracic aorta, case 3. Fatty streak. A large endothelial inclusion exhibits an outer moderately dense layer and an inner very dense material with artifactual striations. The cytoplasm contains an abundance of rough-profiled endoplasmic reticulum. A mitochondrion is well preserved in spite of the 4 hour postmortem period. Along the cytoplasmic membrane are numerous vesicles. Formalin-osmium fixation, 4 hours post mortem. × 15,000.



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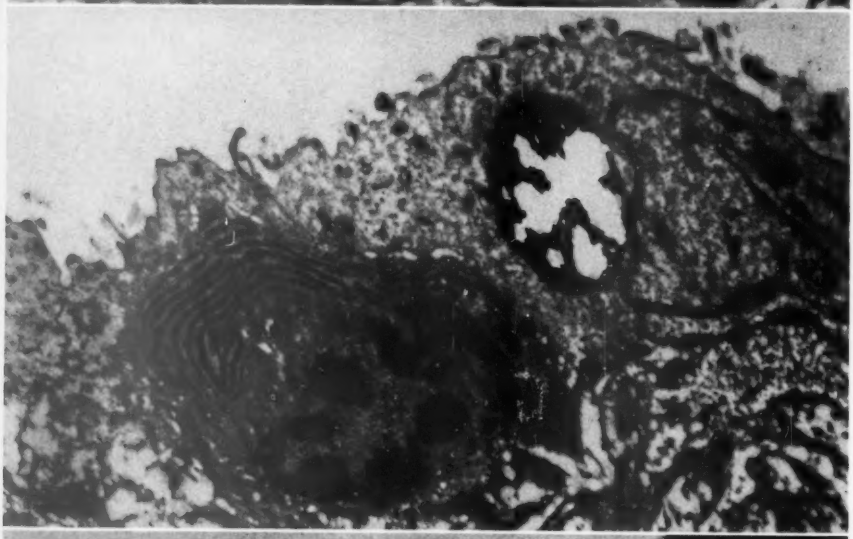

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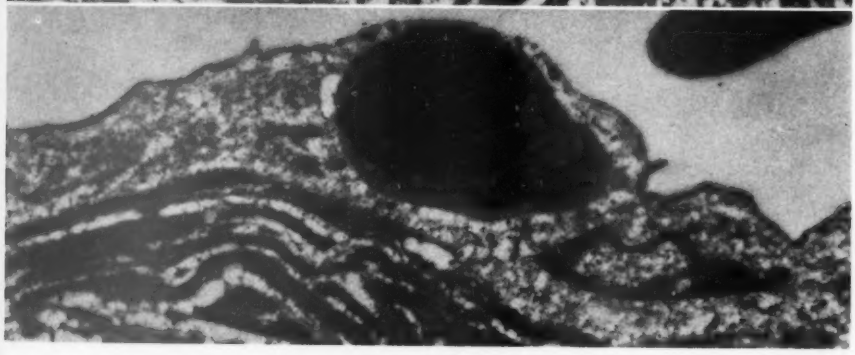

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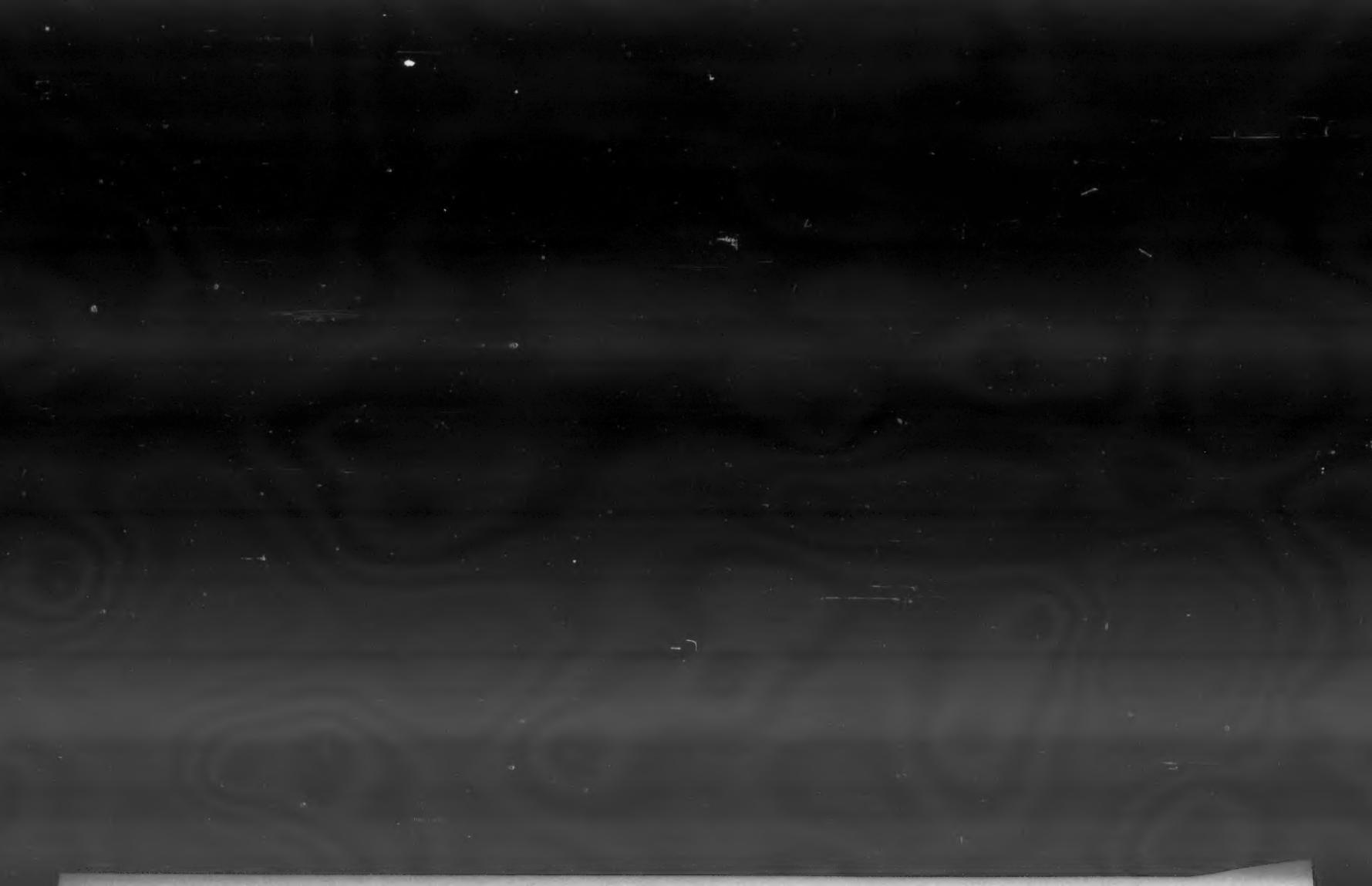
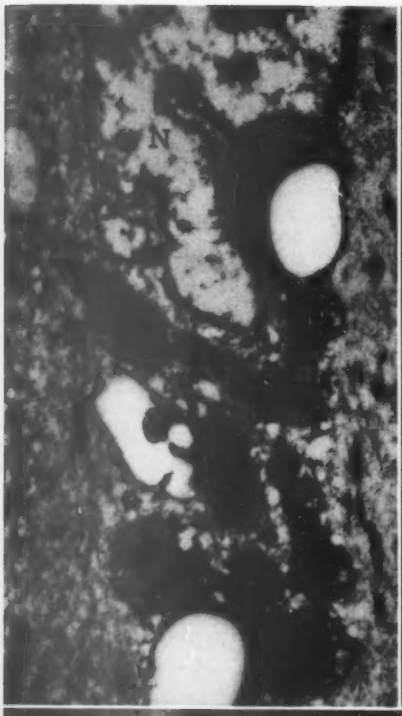
FIG. 6. Human thoracic aorta, case 10. A smooth muscle cell in a fatty streak. The cell contains myofilaments. Lipid inclusions with varying appearance may be noted below and to the right of the nucleus (N). Some have central dense zones with fine filaments and others, large eccentric clear vacuoles. Formalin-osmium fixation, 4 hours post mortem. × 12,600.

FIG. 7. Human thoracic aorta, case 10. A fatty streak showing reticulated lipid inclusions in smooth muscle. The inclusions (I) are composed of a network of dense strands. Two well preserved mitochondria are shown in the upper right, and between the reticulated inclusions lies a swollen mitochondrion. There is no evidence that the reticulated inclusions represent an alteration of mitochondria. Myofilaments identify the smooth muscle cell. The nature of the large clear vacuole (v) with a double limiting membrane is uncertain. Formalin-osmium fixation, 4 hours post mortem. × 53,680.

FIG. 8. Human thoracic aorta, case 8. A fatty streak with a reticulated lipid inclusion in smooth muscle. The inclusion exhibits a central dense area with fine strands radiating toward the periphery. The arrow indicates a double limiting membrane. Formalin-osmium fixation, 1 hour post mortem. × 55,200.





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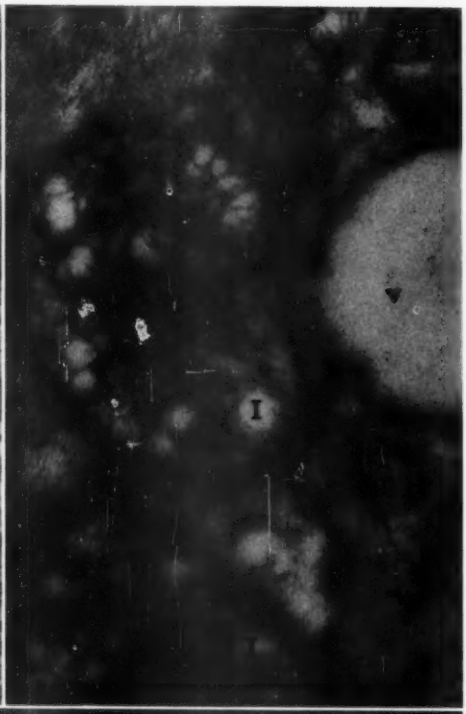

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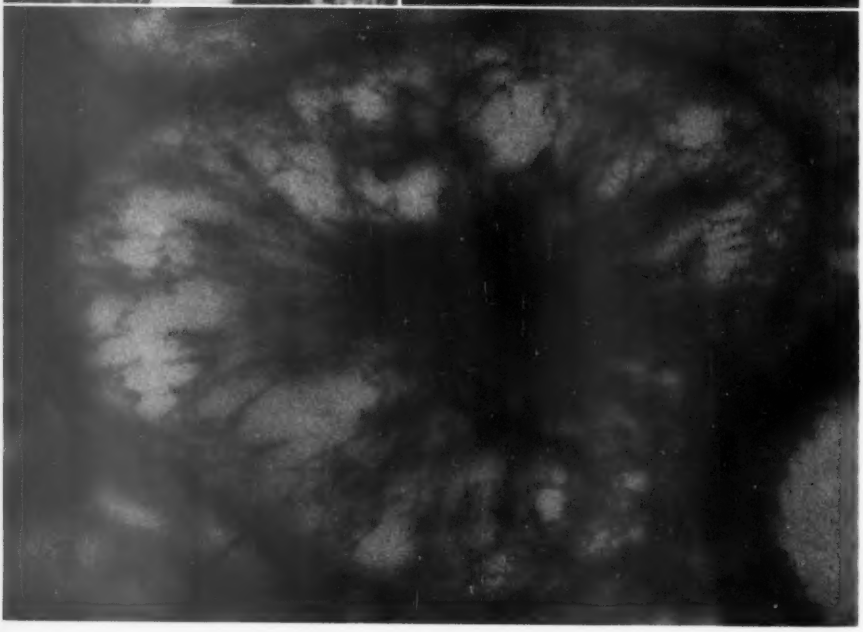

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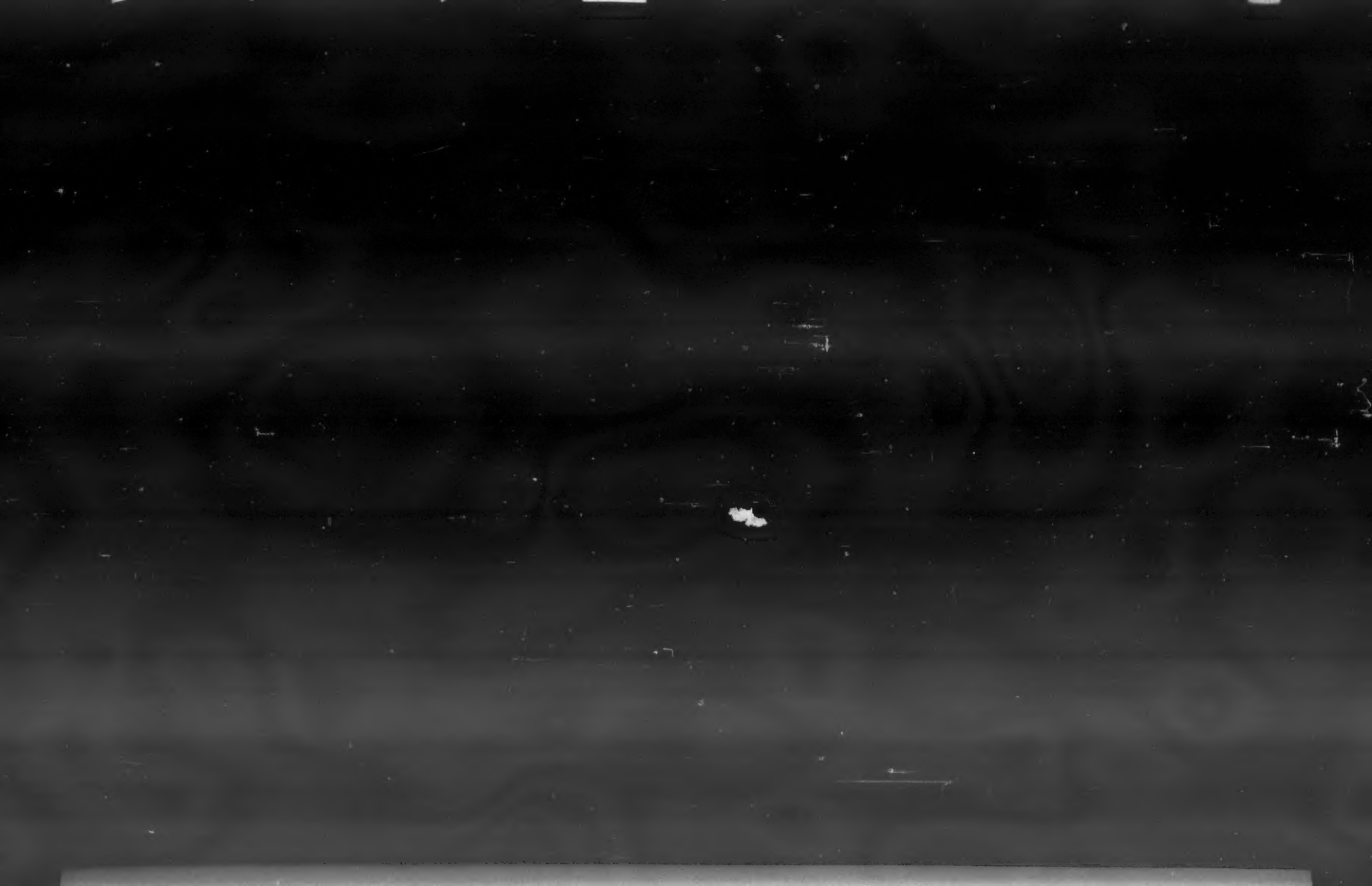
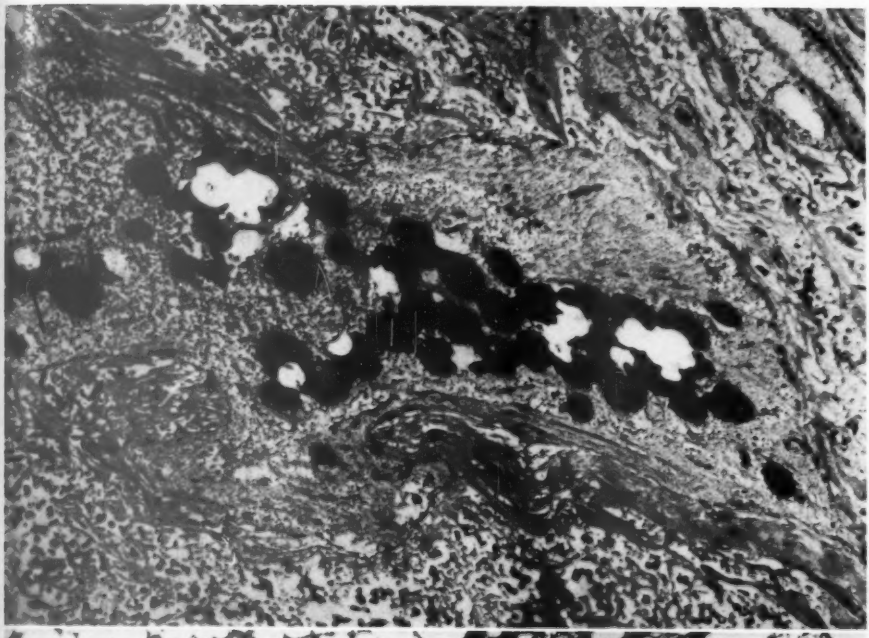
FIG. 9. Human coronary artery, case 5. A fatty streak with dense homogeneous lipid inclusions in smooth muscle. Some inclusions contain clear areas which are usually eccentric (arrow). Among the collagen and elastic fibers in the interstitial space there are numerous small dense bodies. Formalin-osmium fixation, 2 hours post mortem. × 5,250.

FIG. 10. Human abdominal aorta, case 7. Fatty streak. A foam cell is located just beneath the endothelium. The cytoplasm is composed of clear vacuoles with no recognizable organoids. The nucleus (N) is indented by the vacuoles. × 2,750.





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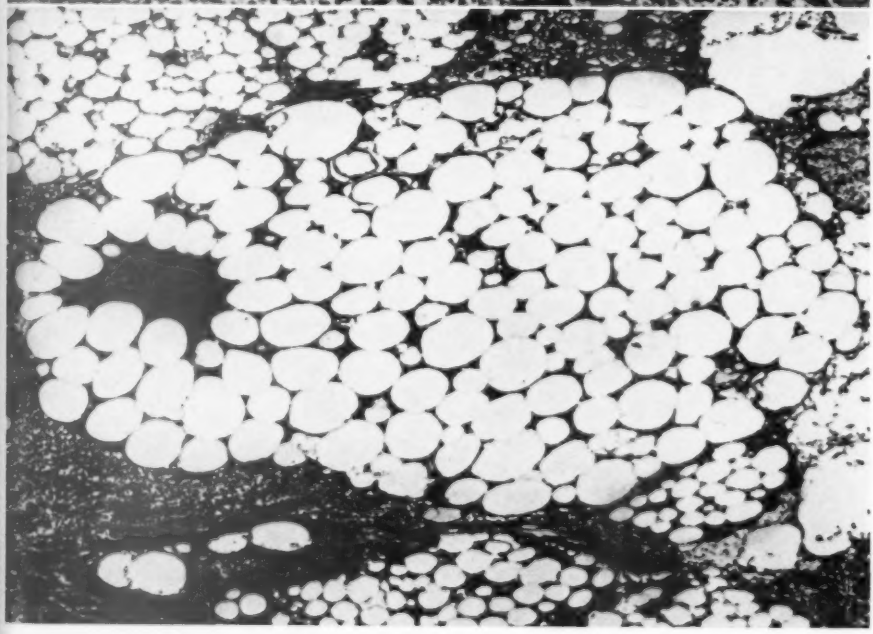

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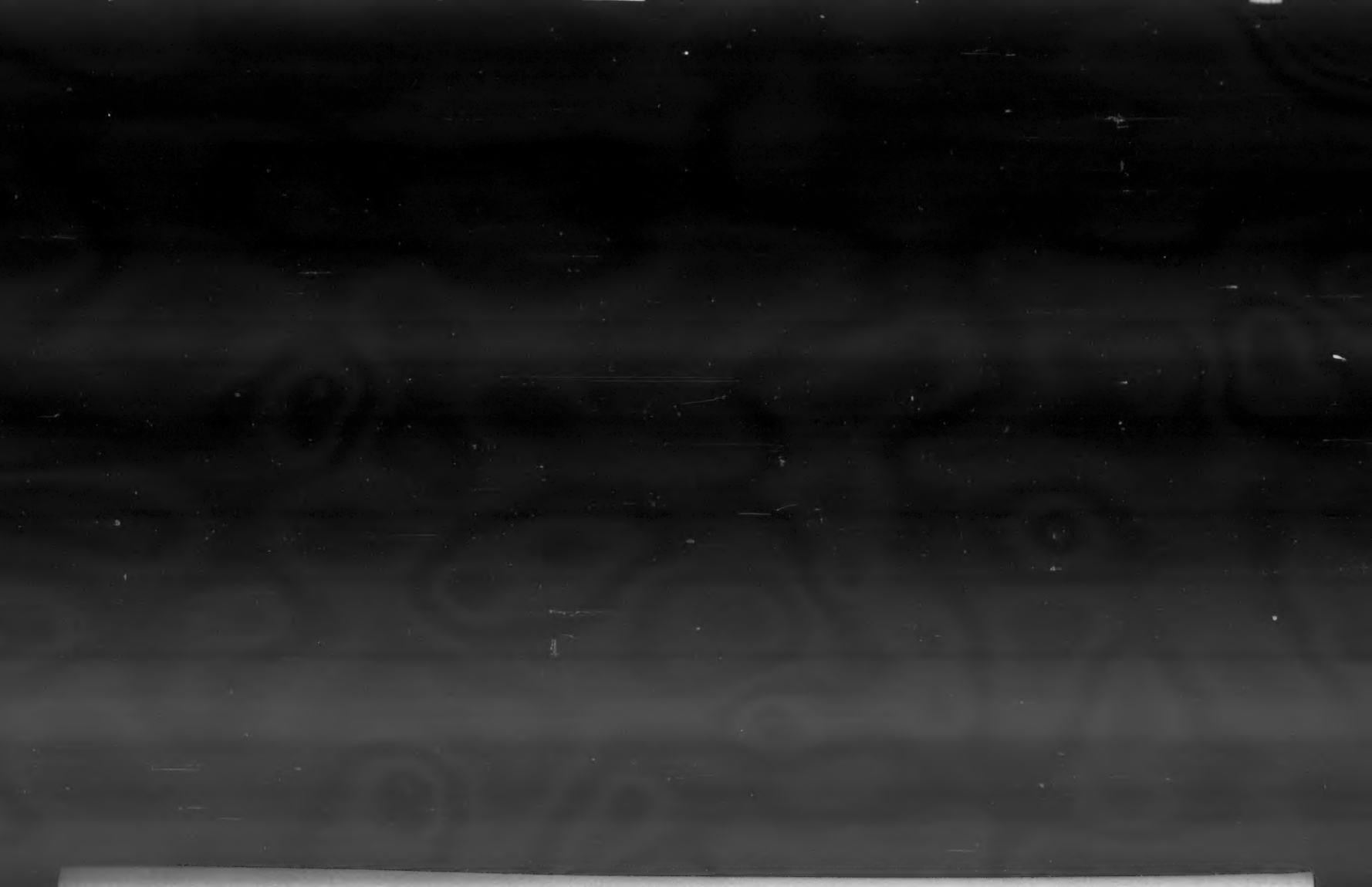
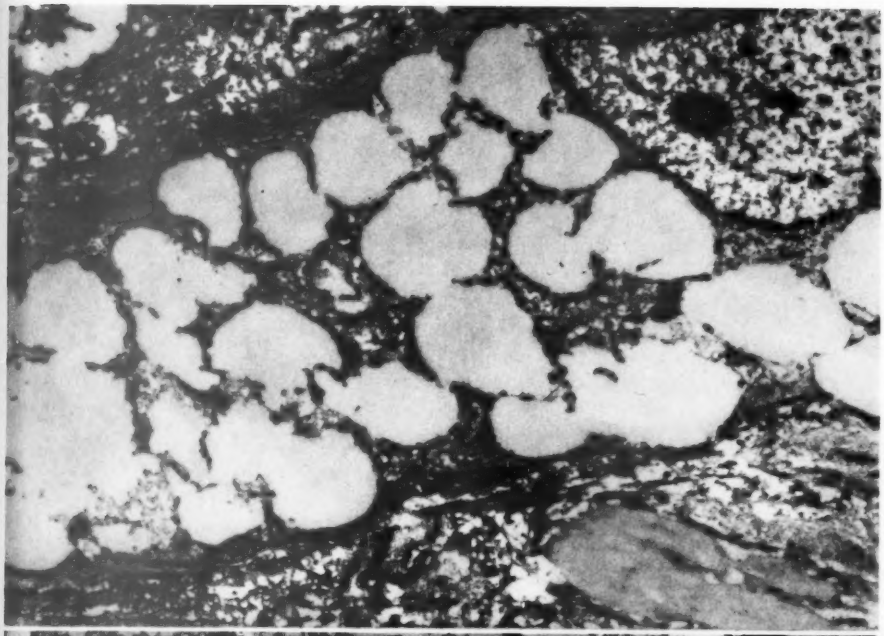
FIG. 11. Human abdominal aorta, case 3. A fatty streak with clear vacuolar lipid inclusions in a smooth muscle cell. The vacuoles are so numerous that the cell appears to be in transition between recognizable smooth muscle and a foam cell. Myofilaments along the cytoplasmic membrane (arrows) identify it as smooth muscle. Formalin-osmium fixation, 4 hours post mortem. × 6,870.

FIG. 12. Human aortic arch, case 1. Fatty streak. Clefts in the interstitial tissue correspond to cholesterol clefts in the carbowax-embedded sample. Dense granular bodies (arrow) are probably calcium. Formalin-osmium fixation, 1 hour post mortem. × 3,740.





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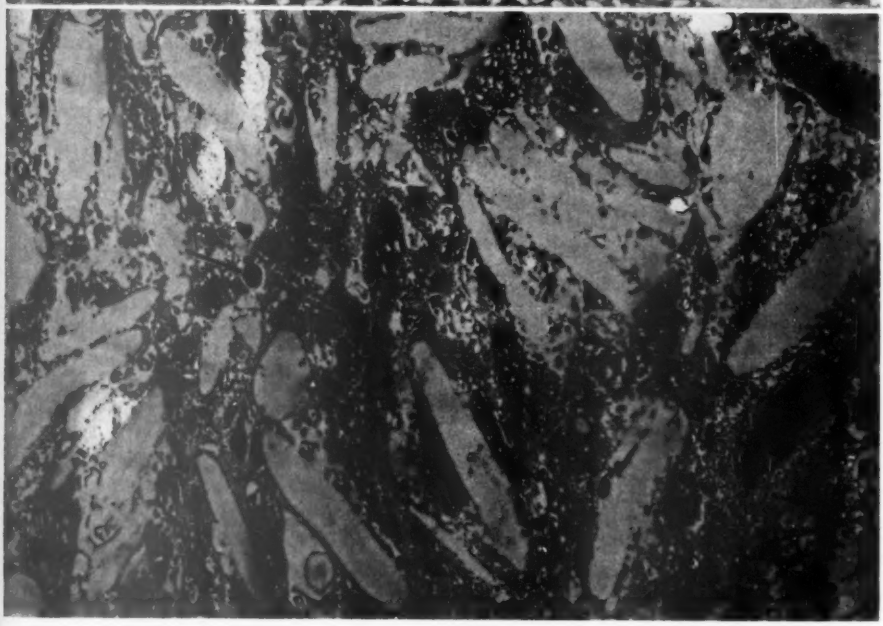

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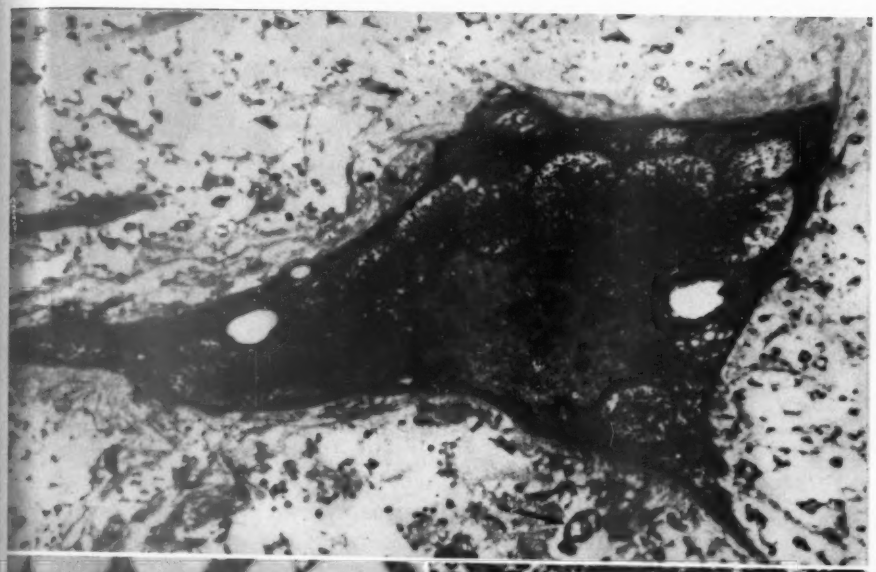
FIG. 13. Human common iliac artery immediately distal to the aortic bifurcation, case 1. An unidentifiable cell containing lipid inclusions and with dense granular cytoplasm appears in a fibrous plaque. In the interstitial region there are numerous small dense bodies with clear centers. At the arrow is a small "myelin form." Formalin-osmium fixation, 1 hour post mortem. × 7,400.

FIG. 14. Human thoracic aorta, case 2. Fatty streak. A foam cell containing a "myelin form." Formalin-osmium fixation, 4 hours post mortem. × 16,800.

FIG. 15. Human abdominal aorta, case 3. Fatty streak. The nature of the moderately dense inclusions (arrows) in this foam cell is uncertain. They probably correspond to PAS-positive droplets in contiguous carbowax sections. The clear vacuoles represent sites once occupied by lipid. Note the crystalline clefts, especially at the lower right. Formalin-osmium fixation, 4 hours post mortem. × 9,000.



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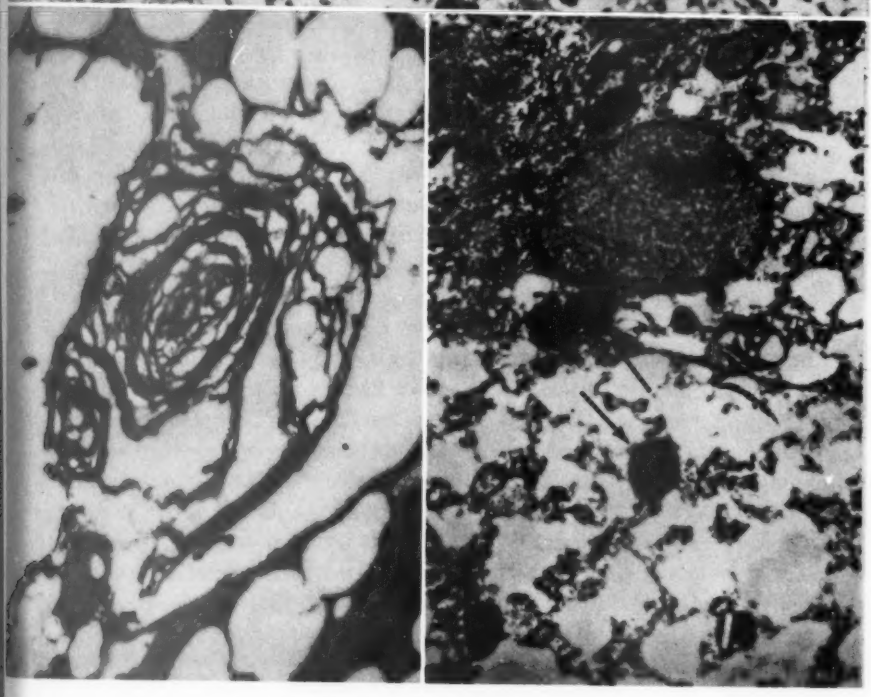

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EXPERIMENTAL ATHERO-ARTERIOSCLEROSIS DUE TO CALCIFIC MEDIAL DEGENERATION AND HYPERCHOLESTEREMIA

GEORGE M. HASS, M.D.; RICHARD E. TRUEHEART, M.D., AND ANNE HEMMENS, B.S.

From the Division of Pathology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Athero-arteriosclerosis varies in type and degree among different people, in different arterial systems and in different branches of the same arterial system. Microscopic changes are principally degenerative and reparative.[1] These changes are often complicated by abnormal accumulations of lipids, deposits of calcium, and events which lead to thrombosis.[2] One approach to an analysis of the pathogenesis of the human disease involves an attempt to reproduce its various forms in animals. The first fruitful attempt disclosed that dietary hyperlipemia and hypercholesteremia in rabbits resulted in the appearance of atheromatous arterial intimal plaques similar to those encountered at times in human disease.[3] However, the degenerative and reparative reactions so commonly associated with the lesions in man did not develop in the experimental atheromatous process. Nor was the distribution of lesions in the experimental disorder similar to that usually encountered in man.

A second type of experimental approach disclosed that certain types of local injury in the arterial wall led to degenerative and reparative reactions similar to those encountered in human arteriosclerosis.[4] A third type of experiment involved an analysis of the combined effects of dietary hypercholesteremia, hyperlipemia and local injury in the arterial wall. This study showed that lipids accumulated electively at the sites of injury, provided there was a reparative mesenchymal reaction, principally in the form of active intimal proliferation.[5] It was also clear that the accumulation of lipids had 3 effects on the local reaction. The first effect was to retard mesenchymal repair; the second was to convert the newly formed mesenchymal structure into hyaline material, and the third was either to inhibit the deposition of calcium in the degenerated media or to accelerate its resorption.

Thus, it became apparent that the reproduction of many microscopic features of human vascular disease required a combination of medial degeneration, mesenchymal proliferation and hypercholesteremic hyperlipemia. With this in mind, it became desirable to investigate generalized

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systemic combinations which might expand the views obtained from local studies. Hyperlipemia and hypercholesteremia combined either with pyridoxine deficiency or hypervitaminosis D appeared as attractive possibilities.[6–8] After a study of hypervitaminosis D in rabbits, it was concluded that it was a suitable method.[9] The disorder had a specific pattern of arterial alteration which was easily reproduced in successive animals. Also, the calcific medial degeneration and subsequent mesenchymal proliferation which characterized the lesions resembled degenerative and reparative reactions in human arteriosclerosis. Hence, various permutations of dietary hypercholesteremic hyperlipemia and hypervitaminosis D were investigated in rabbits. The present report is a description of the results of this study and their interpretation.

Methods

Male albino rabbits, 3 months of age and weighing about 5 pounds, were used.

Irradiated ergosterol dissolved in peanut oil (10^6 U.S.P. units per ml.) was given intramuscularly in doses of 0.1 to 0.15 ml. at daily, biweekly and triweekly intervals in acute experiments. The usual dosage in prolonged experiments was 0.1 ml. twice weekly spaced at intervals of 3 weeks.

The basic diet consisted of Purina rabbit pellets with occasional fresh vegetables. When a cholesterol diet was used, the basic diet was mixed with 0.5 to 3 gm. of cholesterol dissolved in 10 gm. of corn oil in appropriate proportions to make cholesterol concentrations, based on caloric values, vary from 0.01 to 10 per cent.

The following animals were available for study. In the first group there were 70 rabbits kept on a basic diet with viosterol for 1 to 42 weeks. In this group 19 animals were continued on the basic diet for 2 to 24 weeks after cessation of viosterol administration. In the second group there were 56 animals kept on cholesterol diets for 4 to 46 weeks. Among these, 7 animals were continued for 2 to 16 weeks on the basic diet after discontinuing the diet containing cholesterol. In the third group 8 animals were fed cholesterol diets for 4 to 12 weeks and then placed on a basic diet with viosterol regime for 6 to 10 weeks. In the fourth group there were 9 animals fed cholesterol diets for 8 to 32 weeks and given viosterol for 4 to 14 weeks during the latter part of the period of continuous cholesterol feeding. In the fifth group there were 12 animals on a basic diet with viosterol regime for 1 to 24 weeks. Of these, 6 animals were kept thereafter on a cholesterol diet with no viosterol for 1 to 16 weeks and the remainder on a cholesterol diet with continuation of the viosterol regime for 2 to 15 weeks. In the sixth group there were 11 animals kept on a continuous cholesterol diet with viosterol regime for 1 to 22 weeks.

Blood was drawn by cardiac puncture at intervals of 2 to 4 weeks. The serum was analyzed for total cholesterol, calcium and inorganic phosphorus.

Complete necropsies were made on all animals. Tissues from all organs and standard transverse segments of the aortic arch, upper abdominal aorta and lower abdominal aorta, as well as of the iliac, femoral, renal, common carotid and brachial arteries were fixed in 4 per cent formaldehyde (U.S.P.). After fixation, sections were stained for microscopic examination with hematoxylin and eosin and, when indicated, with Scharlach R.

Results

Vascular Disease Due to Hyperlipemia and Hypercholesteremia

The principal initial arterial lesion was an accumulation of lipids in the intima of certain parts of the circulatory system. This was manifest

in part by a concentration of lipids in the cytoplasm of endothelial cells. As these cells acquired increasing lipid content, they tended to become spherical and delicately vacuolated. Soon, additional cells with similar characteristics appeared in the vicinity, and a xanthomatous plaque or mound of lipid-laden "foam cells," presumably largely of mesenchymal origin, was built up (Fig. 1). The base of this mound usually lay on the original subendothelial stroma, and the summit of the mound encroached upon the lumen of the vessel.

The primary xanthomatous intimal plaque tended, thereafter, to undergo spontaneous deterioration despite persistent or rising blood lipid levels. The "foam cells" degenerated and released their contents to form a common, relatively acellular, interstitial pool of lipids enmeshed in a loose fibrillar network. This confluent mass slowly underwent further deterioration with precipitation of cholesterol and its esters in crystalline form and minor deposition of calcium. In this curious degeneration of the primary xanthomatous plaques, certain conspicuous features constantly recurred (Fig. 2). First, there was very little stroma production and no vascularization, so that fibrous intimal plaques comparable to those found in human disease never developed. Second, the stromal reactions in the media were negligible in view of the severe intimal degenerative lipidosis and subintimal or medial xanthomatous changes which commonly accompanied the intimal lesions. Third, the deposition of calcium either in the degenerated intimal plaques or in the subjacent lipid-laden media was minimal. Finally, despite the frequent appearance of excessive amounts of lipid between and in cells of the media, the structure of the media was rarely affected by the sequences of degeneration, calcification and vascularizing mesenchymal reactions so characteristic of human disease.

The anatomic distribution of the intimal atheromatous lesions indicated that each part of the arterial system had a different susceptibility or resistance to the disorder. The lesions began at the root of the aorta and progressed in the form of isolated plaques which tended to enlarge and become confluent. The progression was in the direction of the blood flow, with the process avoiding the small aortic branches and extending more readily into the larger branches. Most of the peripheral arterial system was ordinarily unaffected or very resistant, though considerable alteration was common in the distal small arteries of the coronary, splenic, bone-marrow, gastric and skeletal muscular systems. The pulmonary arterial tree, though less susceptible, was affected in somewhat the same way as the aorta. The lesions were often pronounced in the main pulmonary artery and diminished in the successively smaller branches but never involved the arterioles or the efferent venous system. The cerebral arteries and some other systems were absolutely resistant.

The impression was gained that the resistance of a given arterial system was not only lessened by increasing duration and the level of the hypercholesteremia but also by the extent to which the intima of more susceptible arteries was occupied by lipid deposits.

Vascular Lesions Due to Hypervitaminosis D.

In hypervitaminosis D, the primary arterial lesion occurred in the media as a focus of degeneration and calcification (Figs. 3 to 10). The earliest mesenchymal change incidental to calcification was an apparent increase in the ground substance with a separation of collagenous and elastic fibrils. The earliest cellular changes were in smooth muscle cells, which often became swollen and pale. The earliest calcific deposits were almost ultramicroscopic dustlike particles which became condensed and aligned along fibers and surfaces. The elastic tissue had the greatest affinity for calcium, but the processes, surfaces and internal structure of smooth muscle cells also offered a strong attraction to the mineral. The relative affinities of various structures for calcium and the location of the primary degenerative changes varied from one vessel to another and even from place to place in the same vessel. In general, however, medial calcific degeneration began either in the internal elastic membrane, in the tissues just beneath the membrane or at some slightly deeper level in the inner half of the media. With increasing duration and severity, the foci of calcific degeneration, irrespective of position, gradually increased in dimensions, became confluent and eventually spread to involve the entire thickness and circumference of the media.

At times the medial calcification developed without excitation of any mesenchymal reaction, but usually there was evidence of repair. The earliest sign of repair was in the form of a mesenchymal reaction which constructed a well oriented intimal plaque composed of fibroblasts, a myxomatous ground substance and an endothelial covering. As the plaque matured, collagen and elastic fibrils appeared; soon, collagen became the principal component. The plaque appeared only following subjacent medial degeneration, but this degeneration alone was not always a sufficient stimulus for intimal proliferation. In general, the rate of medial degeneration, the development of structural discontinuities in the course of it and the part of the vascular system affected were the more obvious determinants of intimal proliferation. Suffice it to say that inflammation was not required, and medial degenerative-calcific changes alone were frequently followed by fibro-cellular thickening of the intima equal to the total thickness of the original arterial wall.

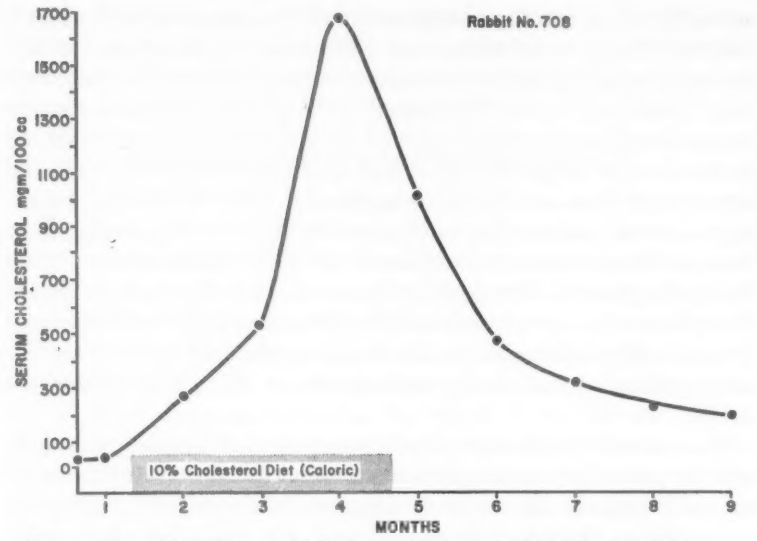
Mesenchymal reactions were not restricted to the intima but also often developed in the media and even the adventitia. These reactions were

ordinarily late in making an appearance. They were associated with repair and resorption of areas of medial calcific degeneration. Usually, resorption was sluggish and essentially without reaction; at times, however, it was very active. The principal stimulus to resorption was the encroachment of the medial lesion on the adventitia. This activated the proliferation of adventitial fibroblasts which penetrated the media and carried with them a network of capillaries. The vascularized mesenchyme spread around the medial calcific deposits. Macrophages, at times multinucleated, were mobilized. The degenerated calcified tissue slowly disappeared. Occasionally, bone was formed, but as a rule the resorption was so complete that the resultant vessel wall was composed of a thick fibro-cellular intima and a thick adventitia bound together by a thin sheet of vascularized granulation tissue which had replaced the media.

The anatomic distribution of the lesions indicated that each part of the arterial system had its own particular susceptibility or resistance to the metabolic disorder. The lesions first appeared at the root of the aorta and progressed in the direction of the blood flow, avoiding small aortic branches and extending more readily into the larger branches. Most peripheral arterial systems were less severely involved though the smaller divisions of special arterial systems were regularly affected. Among these, the small arteries and arterioles supplying the inner third of the renal cortex, the acid-secreting part of the stomach, the duodenum, the salivary glands and skeletal muscle were most conspicuous. Other systems were less susceptible, and the lesions became evident only when systems with a greater susceptibility had undergone calcification. It seemed that the circulating calcium sought the most elective site and accumulated there, but if this site was occupied by prior deposits, deposition occurred by second or random choice, as it were, in the next most elective site. Some systems were absolutely refractory to the disease. Hence, the anatomic distribution of calcific medial degeneration with fibrous intimal proliferation due to hypervitaminosis D differed from that of intimal lipidosis due to hypercholesteremia. The difference was particularly conspicuous in instances where the distal arteries and arterioles, severely affected by the calcific process and resistant to intimal lipidosis, supplied structural units co-ordinated in the performance of certain special functions.

Vascular Lesions Due to Combinations of Viosterol Administration, Hyperlipemia and Hypercholesteremia

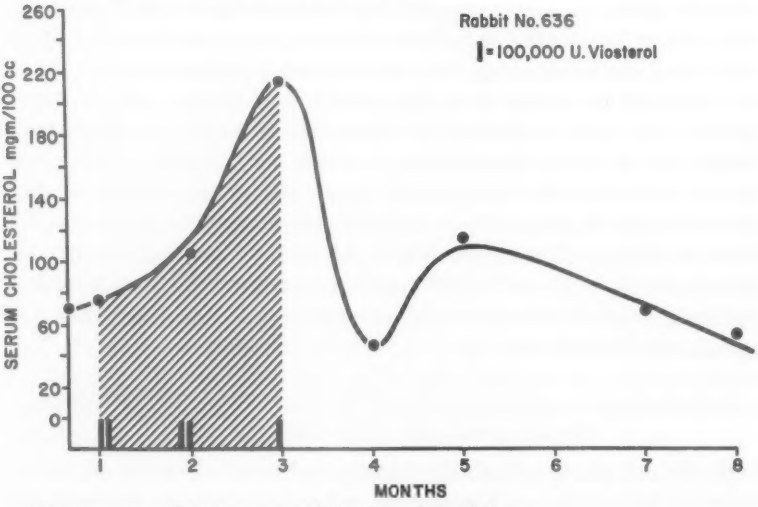
Rabbits on a normal basic diet usually had serum cholesterol levels of about 50 to 75 mg. per hundred cc. After institution of the viosterol



TEXT-FIGURE 1. Normal rise and fall of serum cholesterol with cholesterol diet.

regime and without change in diet, the levels usually rose within 4 to 12 weeks to about 150 to 250 mg. and occasionally to 400 mg. per cent (Text- fig. 2). Later on, the levels tended to fall to 100 to 150 mg. The decline ordinarily was accompanied by loss of weight and often occurred despite progression of arterial and renal disease.

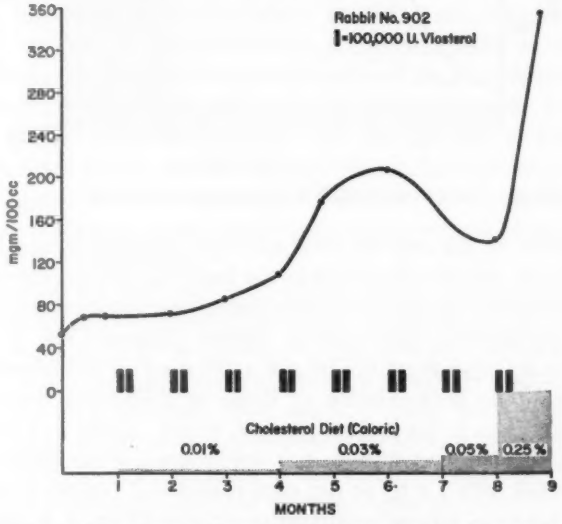
When normal rabbits were given a dietary supplement of 1.5 to 10



TEXT-FIGURE 2. Rise in serum cholesterol on viosterol dosage.

per cent cholesterol (caloric) with corn oil, the serum cholesterol levels usually rose within 4 to 12 weeks to 1,000 to 3,000 mg. and occasionally to more than 4,000 mg. (Text-fig. 1). Superposition of the viosterol regime on a diet with as little as 0.25 per cent cholesterol (caloric) led to rapid extreme elevations of serum cholesterol (Text-fig. 3). This effect was not as pronounced in hypercholesterolemic animals if they were on a cholesterol-free diet during the period of viosterol administration (Text-fig. 4).

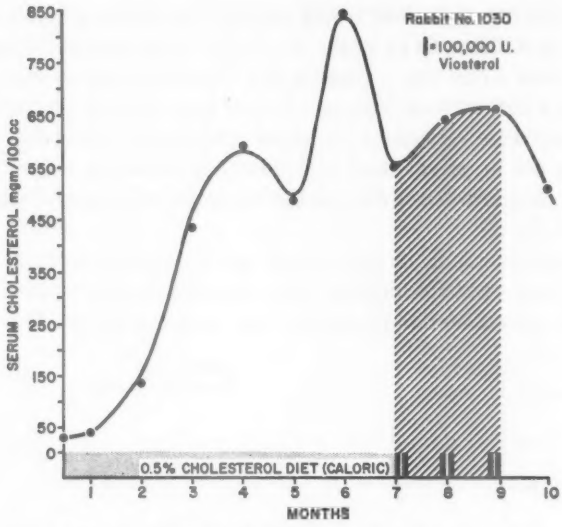
Hypercholesteremia in the various groups of experimental animals, therefore, had 3 possible origins; first, dietary; second, viosterol action; and third, a combination of dietary and viosterol action. It was noted



TEXT-FIGURE 3. Rising serum cholesterol with viosterol and dietary cholesterol.

that animals with dietary hypercholesteremia usually had, in addition to the customary atherosclerosis, widespread xanthomatous mesenchymal reactions unrelated to the atherosclerotic process. Animals with viosterol-induced hypercholesteremia developed xanthomatous reactions only in connection with the arterial lesions. Animals with a hypercholesteremia of mixed origin had a tendency to localize the xanthomatous reactions where mesenchymal activation accompanied arterial and extra-arterial lesions produced by hypervitaminosis D.

Calcific medial degeneration of many arterial systems was conspicuous after 4 to 8 weeks of viosterol administration (Figs. 3 to 7). The degenerative change often stimulated fibro-cellular proliferation of the overlying intima; this was ordinarily most active from the fourth to the



TEXT-FIGURE 4. Viosterol dosage during falling serum cholesterol.

eighth weeks. If the average level of serum cholesterol was as low as 150 to 200 mg. during this period on the basic diet with viosterol regime, local accumulation of lipids and lipid-laden mesenchymal cells occurred in some proliferating fibro-cellular intimal plaques (Fig. 5). At this level of serum cholesterol the intima uninvolved by proliferative reactions showed no accumulation of lipids or lipid-laden macrophages. Lipid localization in the intimal plaques, which formed as a reaction to subjacent medial degeneration, was accentuated if the basic diet was supplemented with 1.5 to 10 per cent cholesterol (caloric) during the period of maximal intimal proliferative activity (Figs. 3, 6 and 7). The dietary lipid supplement usually led within 2 to 8 weeks to a prompt rise of serum cholesterol to 1,000 mg. or more. Within 7 to 21 days after institution of the supplement, while serum cholesterol levels were rising to 350 to 500 mg., lipids and lipid-laden mesenchymal cells became conspicuous in many actively proliferating fibro-cellular intimal plaques (Fig. 7). During this period no accumulation of lipid was found in the intima of any artery unless it was affected by medial calcification and secondary mesenchymal reactions. A significant degree of lipidosis in the intimal vessels without medial lesions ordinarily did not occur in the viosterol-treated or control animals on a cholesterol diet until they had been on the diet for at least 12 to 22 weeks with average serum cholesterol levels much higher than 350 to 500 mg.

A number of animals were kept on the cholesterol diet for several

weeks after stopping viosterol administration. In these, progressive intimal lipidosis did not follow the pattern characteristic of a prolonged purely hypercholesteremic state. Complex reparative mesenchymal reactions incidental to medial calcification, now in its regressive stage, continued to influence the localization of lipid deposits (Figs. 8 and 9); this influence was not always favorable to deposition. For instance, lipid deposition and macrophage accumulation were retarded wherever normal intima or dense inactive fibrous plaques overlay broad zones of unresolving quiescent calcific medial degeneration (Fig. 9). On the contrary, conditions favored continuous elective localization of lipid wherever mesenchymal reactions continued. Frequently, in the course of several months, the mesenchymal activity followed a characteristic pattern (Fig. 9). In the beginning, it was confined principally to the intima. Somewhat later, as regression and resorption of the medial lesions began, the activity shifted from the intima to the media. As repair of the medial process became increasingly active, the mesenchyme in the adjacent quiescent thick fibrous intima was reactivated, and intimal plaques underwent further proliferative changes. In each instance, lipid localization and accumulation of "foam cells" followed the pattern of mesenchymal activation and reactivation.

It was curious that although mesenchymal activity secondary to medial calcification was conducive to lipid accumulation, the subsequent localization of lipids in the intima or media retarded further progression of the viosterol-induced processes which elicited the activity initially. Evidence for this inhibitory effect was of 3 types. First, medial calcific lesions seldom developed in the arterial wall beneath thick intimal lipid plaques (Fig. 4). This protective effect seemed due not only to the trapping of calcium as it diffused from the blood through the plaque but also to a shift in the equilibrium to favor local resorption rather than deposition of calcium. Also, resorption of calcified matrices, though accelerated by local stromal penetration, vascularization and mobilization of macrophages, also was enhanced by lipid accumulation. The second means by which lipid accumulation retarded progression of the viosterol-induced process was by action as a deterrent to progressive formation of collagen and new capillaries. This was attributed not only to conversion of potential collagenogenic fibroblasts and capillary endothelium to lipid-laden "foam cells" but also to the deleterious local effect of excess lipids. The latter seemed to combine with intercellular fibrils and interfere with their proper formation for support of vascularization. This effect was characterized by hyalinization of collagen or elastic tissue with abnormal fusion and fraying of fibrils. The third means of interference with the development of lesions due to hypervitaminosis D

was apparently attributable to operation of systemic mechanisms. It was clear that at a standard viosterol dosage level the hypercholesteremic state increased the animal's tolerance to viosterol and retarded the development of calcific degenerative changes in many locations. This was conspicuous in the tubules of the kidneys, in the smooth muscle of the stomach and in cardiac or skeletal muscle cells. In other locations, especially the broncho-alveolar system, the opposite effect was noted and hypercholesteremia enhanced calcific degeneration. Though the distribution of enhancing effects was not always the same in the various tissues, it was clear that the hypercholesteremic state had a systemic as well as a local influence in reducing or increasing calcification potentials of many tissues.

The antagonisms and synergisms between mechanisms governing hypercholesteremic and viosterol-induced vascular lesions in the same animal were also apparent when dietary hypercholesteremia was produced several months after the institution of viosterol administration. In these animals the viosterol-induced disorder had long since reached its maximal development and was usually stationary or slowly regressing. This late indolent stage of disease had quite a different influence on lipid localization than the earlier active stage (Fig. 9). For instance, mature, quiescent, thick fibrous intimal plaques overlying dense calcific medial lesions, especially in the aortic arch, were more resistant to lipid deposition than adjacent normal intima. Lipids which accumulated in the fully formed fibrous plaques seemed to fuse with the collagen to form compact hyalinized fibrous tissue and failed to provoke a conspicuous aggregation of lipid-laden cells. On the contrary, multiple layers of lipid-laden cells were electively accumulated to form thick nonfibrous atheromatous plaques in the near-by "normal" intima overlying medial structure unaffected by calcific degenerative changes. These observations were consistent only in the proximal aorta. Elsewhere, lipid localization continued to be governed largely by the relative mesenchymal quiescence and activity in viosterol-induced vascular lesions. This was especially notable in arterial systems ordinarily resistant to hypercholesteremic intimal lipidosis but susceptible to viosterol-induced medial calcific lesions. When local mesenchymal activity was sufficient either in the course of progression or in reparative regression of lesions, lipid localization occurred. At times, this produced bizarre forms of xanthomatous arteritis involving small vessels. When the xanthomatous reactions were in the form of an endarteritis, hyalinization of the internal elastic membranes with resorption of elastic tissue and calcium from the subjacent inner media was conspicuous. Various combinations of xanthomatous endarteritis, mesarteritis and periarteritis also oc-

curred. These combinations favored demineralization of small arterial and arteriolar walls so that they finally were converted to concentric rings of elongated lipid-laden macrophages between which remnants of resorbing calcified medial structure were still recognizable (Fig. 10).

Experiments in which dietary hypercholesteremia existed for 4 to 8 weeks prior to supplementing the regimen for 4 to 8 weeks with viosterol also served to illustrate the antagonistic and synergistic actions of hypervitaminosis D and hypercholesteremia. As in other experiments, the result of the combined regimes depended upon the sequences and severity of the development of hypercholesteremic and viosterol-induced lesions.

A regimen productive of almost pure vascular disease with specific localization of lipids in viosterol-induced lesions and minimal changes in extravascular tissues was as follows: 10 per cent cholesterol diet (caloric) for 4 weeks; 1.5 per cent cholesterol diet (caloric) and 400,000 units (U.S.P.) of viosterol during the next 6 to 8 weeks; normal diet and no viosterol for a terminal recovery period of 6 to 8 weeks. The reasons for the result were as follows: First, the existing state of hypercholesteremic hyperlipemia opposed the tendency of viosterol to promote calcium deposition, especially within parenchymal cells of many extravascular tissues. Second, the period of early active formation of lipid plaques on the hypercholesteremic regime (12 to 18 weeks after institution of the cholesterol diet) coincided with the period of maximal activity of intimal fibro-cellular reactions due to hypervitaminosis D (4 to 8 weeks after institution of viosterol dosage). Third, the terminal rest period of 6 to 8 weeks was sufficient to allow the subsiding hypercholesteremic state to add an element of chronicity to the disturbance, to reduce the magnitude of the extravascular calcific disease through enhanced resorption and to allow completion of regeneration of damaged extravascular tissues. These experiments disclosed also that intimal plaques of lipid-laden cells protected the subjacent media against calcific lesions even though the plaques displayed no affinity for calcium unless they were degenerative. This was in keeping with the occurrence of calcification in plaques of hyperlipemic animals on a cholesterol diet without supplementary viosterol.

When the period of hypercholesteremic hyperlipemia was more prolonged (7 weeks or more) before the animal was placed on a basic diet with viosterol, the type of vascular disorder was again modified. This regimen tended to accentuate the protective action of intimal lipid deposits so that medial calcific lesions in the proximal aorta were minimized (Fig. 4). Also, it accentuated the accumulation of lipids in the distal aorta and branches where the viosterol-induced lesions excited mesenchymal activation. Thus, lipids accumulated beyond the region of

lipid deposition characteristic of hypercholesteremia alone. Severe forms of athero-calcific disease in distal arteries, especially in skeletal muscle, occurred in animals on this regimen. At the same time, athero-calcific lesions were minimal in the proximal aorta. It was clear that atheromas continued to form over viosterol-induced lesions even though the animal was on a cholesterol-free diet with levels of serum cholesterol rapidly falling from about 1,000 mg. per hundred cc. to 150 to 250 mg. (near the high level for animals on sustained viosterol dosage). The level below which no further lipid deposition occurred was not established precisely, but it was about 150 to 200 mg. It was of interest in this connection that viosterol administration, though accompanied by sharp elevations of serum cholesterol when there was cholesterol in the diet, was unable to sustain the high levels after the animal was placed on a normal diet. It was inferred from this that viosterol was not effective in mobilizing the extensive widespread lipid accumulations in animals with chronic severe hypercholesteremia.

When a prolonged dietary hypercholesteremia (20 weeks or more) existed prior to viosterol administration, the antagonism between lipid deposits and the development of viosterol-induced disease was more clearly shown. In these animals, calcium was deposited principally in degenerated intimal atheromas and in the media of arteries where there was no overlying protective atheromatous plaque. It was again demonstrated that in the distal aorta and major branches, atheromas which developed together with or just subsequent to the medial disease enhanced the local resorption of calcium from the subjacent media. More distally, in the small arteries, especially in skeletal muscle, mixtures of athero-calcific disease were conspicuous.

The more prolonged the life of the animal during or following the viosterol regime, the more outstanding were the complex reparative and resorptive processes in the media of degenerated calcified vessels. These processes were recognizable everywhere but were most easily analyzed in the lower aorta and iliac or femoral arteries. Following the usual initial stages of the medial degenerative calcific sequences, there was stimulation of fibro-cellular intimal proliferation. The progress and magnitude of the proliferation depended upon the vessel affected, the activity of the medial lesions and other still unrecognized factors. The progressive formation of fibro-cellular intimal plaques was unaccompanied by vascularization. Even the infiltration of the plaques with lipids and the accumulation of "foam cells" which often disintegrated failed to stimulate significant vascularization. The sequences of repair in the media, however, were different. As medial calcific degeneration spread from the inner or middle third of the vessel wall toward the adventitia,

there was often an indolent stimulation of mesenchyme. This became more conspicuous as the gap between the wave of medial calcific degeneration and the normal adventitia closed. As closure of the gap by progressive degeneration was completed, adventitial reactions became conspicuous in focal areas and mesenchymal penetration of the adjacent degenerated media slowly developed. As the proliferating mesenchyme penetrated the media, it was often accompanied by small capillaries, and when this occurred, local resorption of calcified medial structure was accelerated. In the hypercholesteremic animals, mononuclear and multinuclear mesenchymal cells laden with lipids appeared in the vascularized stroma and seemed to participate in the mechanism of medial resorption (Figs. 8 and 9). As the calcium deposits and calcified matrix of the degenerated media slowly disappeared, hyalinized fibrous tissue, osteoid tissue and well organized bone often appeared in its place. At other times, the resorption of the media was so complete that the adventitia became fused with the thickened intima which in turn was stimulated to increased fibrous or fibro-xanthomatous thickening by the influence of the proliferating activated subintimal mesenchyme (Fig. 9). In this fashion, reproduction of almost every complex histologic detail of human athero-arteriosclerosis, except for intimal vascularization and occlusive thrombosis, was recognized. Furthermore, the mechanisms leading to the varied microscopic complexes were clearly defined in pictorial sequence of the degeneration and repair influenced by permutations of hypervitaminosis D and hypercholesteremia.

Two important details in the evolutionary sequence of human athero-arteriosclerosis were not reproduced. One missing feature, so conspicuous in human disease, was thrombosis. Thrombosis never occurred despite severe narrowing of arteries with extreme degrees of intimal and medial degeneration. Perhaps the answer to this problem lay in the absence of vascularization of the degenerated plaques, for thrombosis has been produced in monkeys on a long-term hypercholesteremic regime.[10] The other feature was the absence of changes of any kind in some arterial systems often severely affected in human disease and the presence of severe alterations of varied complexity in other arterial systems ordinarily lightly affected in the human subject. For these reasons work with other species and exploration of the influence of conditions in addition to hypercholesteremia, hyperlipemia and hypervitaminosis D on the pattern of experimental vascular lesions are indicated.[11,12] Whatever result may follow, it may be predicted that the basic rules established for relations between medial degeneration and hypercholesteremic hyperlipemia in these experiments will remain unchanged, irrespective of species or etiology of the vascular lesions. The anatomy of distribution

of the disease might vary among the different species, but even this will be in keeping with the rules, whatever the pathogenesis of medial degeneration, hypercholesteremic hyperlipemia or reparative reactions in the arterial wall might be.[11–14]

Quantitative Relations Between Hypercholesteremia and Intimal Atheromatous Deposition

These studies showed that significant lipid deposition in normal arterial intima did not occur even in prolonged experiments unless the average serum cholesterol levels exceeded 350 to 400 mg. per hundred cc. These values are similar to those required for lipid deposition in monkeys.[12] When intimal mesenchymal activation and hypercholesteremia were optimally simultaneous, lipid deposition occurred in some active intimal lesions even in brief experiments when the average serum levels were about 150 to 200 mg. The pattern of deposition under these conditions generally followed the pattern set by the susceptibility of the affected arteries to hypercholesteremia rather than hypervitaminosis D. This susceptibility may be regarded as a lipidosis potential, and the greater the susceptibility, the higher the potential. In other words, with equal intimal activation in the aorta and its major branches due to hypervitaminosis D, the degree of lipid deposition was proportional to that expected of normal intima at more prolonged, higher serum cholesterol levels. When intimal fibro-cellular activation and hypercholesteremia were least optimally simultaneous, lipid deposition in the diseased intima did not occur even at serum cholesterol levels in excess of 500 mg. or at similar levels sufficient to lead to lipid deposition in near-by normal intima. Furthermore, there was good evidence that the average level of serum cholesterol required for maximal lipid deposition in activated intima was less when the serum level was rising than falling. Finally, there was evidence that unpredictable variations were attributable to an acquired tolerance to one or the other regimes. There were doubtless other regulatory factors, but the main point to be emphasized is that the experimental levels of serum cholesterol productive of significant atheromatous accumulation in the presence of degenerative calcific medial disease with secondary active intimal proliferation were not necessarily higher than adult "normal" levels in man. Of still greater significance was the disclosure that brief fluctuations of a few days' duration above "normal" human levels amounting to no more than 50 mg. were sufficient to produce significant lesions in activated intima where otherwise only minimal or no lesions would have occurred. All this lends support to the idea that there may be a close relation between the effects in a given time interval of a given level of serum cholesterol

on a normal or abnormal arterial system, irrespective of diversity of species, diversity of mechanisms responsible for serum cholesterol elevation and diversity of causes of arterial diseases which provide the soil for lipid localization.

Discussion

The first conclusion to be drawn from these experiments was that medial calcific degeneration of an artery in the absence of regional mesenchymal activation was not conducive to lipid localization in the intima or media, despite hypercholesteremic hyperlipemia sufficient to produce localization in near-by normal intima.

The second conclusion was that medial calcific degeneration of an artery was conducive to lipid localization in the intima or media, provided there was mesenchymal activation in these locations and that under optimal conditions, lipid accumulation occurred at levels of hypercholesteremic hyperlipemia well below those required for localization in near-by normal intima.

The third conclusion was that lipid localization in the absence of associated cellular necrosis was antagonistic to calcium deposition. This antagonism was manifest in two ways. Intimal atheromatous lesions protected the subjacent arterial wall from degenerative calcific disease, and the protection was proportional to the thickness of the atheromatous plaques. Medial calcific lesions, once established, underwent accelerated resorption and resolution in the presence of xanthomatous lipid deposits, in either the activated intimal or the medial mesenchyme.

These fundamental relations between degenerative medial calcific disease, the secondary excitation of reparative mesenchymal reactions and the local accumulation of lipids had an important influence on the local and systemic characteristics of combined metabolic disturbances due to permutations of hyperlipemic hypercholesteremia and hypervitaminosis D. Each disturbance, when operating alone, was productive of a specific form of vascular alteration. In the instance of hyperlipemia and hypercholesteremia the lesion was in the nature of an intimal xanthomatosis. It developed initially at the root of the aorta and proceeded with diminishing intensity in the direction of the flow of the blood and thence into the larger branches. The small arteries and arterioles in most systems other than the coronary, gastro-duodenal, pulmonic, splenic, skeletal and myeloid were largely spared. Main muscular arteries such as the brachial, renal and femoral were seldom seriously affected. Some arteries were wholly resistant.

In the instance of hypervitaminosis D, the disease was in the nature of a calcific medial degeneration. This also developed initially at the root

of the aorta and proceeded with decreasing intensity in the direction of the flow of the blood and thence into the larger branches. In contrast to the hypercholesteremic atheromatous disease, large muscular arteries such as the iliac and femoral were severely involved. Furthermore, the distribution of the lesions in the various organ vascular systems was not the same as that in the atheromatous disorder. This was especially conspicuous in the heart, skeletal muscle, salivary glands, kidneys, lungs, stomach and duodenum. The secondary mesenchymal reactions in the form of fibro-elastic intimal proliferation or medial fibrosis with and without vascularization depended upon several factors. The occurrence of degenerative-calcific changes in the vascular wall was an important factor, but the mere presence of these changes was not sufficient to elicit mesenchymal reactions. These reactions were more likely to increase with increasing duration and degree of the medial changes, especially when the continuity of internal or external elastic membranes was disrupted. Also, the reactions were more likely to occur in connection with medial lesions which had unusually rapid rates of development or regression. Finally, the pattern of mesenchymal reactions depended upon the part of the vascular system in which the calcific lesions occurred. In general, intimal fibro-cellular proliferation increased along the aorta and reached maximal relative thickness in the iliac and femoral arteries. Ordinarily, small branches showed little intimal proliferation although at times in special systems this was excessive, leading to extreme narrowing of the lumens of small arteries and large arterioles.

It was not surprising, therefore, that the experimental permutations of hypercholesteremic hyperlipemia and hypervitaminosis D provided a broad spectrum of vascular disorder. At one end of the spectrum was a form which developed after hypercholesteremic atheromatous lesions had been well established before administration of vitamin D. Thick lipid plaques, often in continuity, afforded protection to certain parts of the vascular system. Hence, intimal lipidosis was conspicuous in the proximal aorta while calcific medial lesions were conspicuous in the distal aorta and large branches. Also, calcific medial disease failed to develop in peripheral coronary, gastric and splenic systems replete with intimal lipid deposits. In this disorder, the presence of hyperlipemic intimal lesions led to a marked modification of the usual pattern of calcific degeneration, tending to restrict the development of calcific-degenerative and intimal fibro-cellular proliferative disease to those parts of the vascular system relatively unaffected by hyperlipemic intimal process.

At the other end of the spectrum was a form which occurred when calcific-degenerative lesions with mesenchymal reactions were present

prior to the superposition of the hyperlipemic disease. Active intimal mesenchymal reactions favored localization of lipids while mature fibrous intimal plaques resisted this localization so that the normal pattern of pure hypercholesteremic disorder failed to develop. There was a sharp shift of the deposition of lipids into the peripheral systems of large and small arteries in accordance with the distribution of active intimal and medial mesenchymal reactions. In prolonged experiments the medial mesenchymal reactions were closely related to the resorption of medial calcific deposits, and as the calcific deposits resorbed, conspicuous xanthomatous cells rich in lipids appeared to displace them. At times, however, the resorption of the calcific deposits, especially in the presence of newly formed vascularized stroma, was accompanied by the formation of osteoid matrix and bone. At other times, the eosinophilic matrix that appeared in the background as calcific deposits were resorbed acquired hyaline properties, but only in the presence of hyperlipemia or local lipid accumulation. Similar hyaline changes also occurred in the proliferating intimal lesions.

Between these two extremes, there were many complex patterns of vascular alteration which were the result of the operation of the previously mentioned basic mechanisms in time and in relative degree. Almost every microscopic detail of human athero-arteriosclerosis was reproduced. There was one major exception. Despite the frequency of malnutrition, terminal infection and very severe arterial disease, occlusive thrombosis was not encountered. In other words, we have not found any combination of conditions productive of a metabolic vascular disease which has a tendency to thrombosis as described in other species.[10] Nor were we able to produce a pattern of vascular lesions which had the systemic distribution of human athero-arteriosclerosis, though under special conditions the pattern was surprisingly similar. Thus, we may be justified in speculating that athero-arteriosclerotic thrombosis in man requires conditions which differ qualitatively or quantitatively from those in these experiments and that the medial calcific degeneration in man has a pathogenesis with some features in common with that of calcific medial degeneration produced by hypervitaminosis D. Greater duration may be most important.

An Experimental Basis for a Theory of the Pathogenesis of Human Athero-Arteriosclerosis

There are good reasons for believing that the principal predisposing event in the pathogenesis of most human athero-arteriosclerosis is medial degeneration. There is good microscopic and chemical evidence that medial degeneration usually occurs before the onset of other recognized

changes. There is good evidence that medial degeneration is often a stimulus to mesenchymal reactions in the arterial wall and that these may occur under circulatory conditions as nearly normal as those which obtain under the influence of prolonged changes in peripheral demand. There is no evidence that the usual mesenchymal reactions occur in the absence of medial and subintimal changes. There is no evidence that any other local or systemic alteration is regularly associated with intimal lipidosis or mesenchymal proliferation.

The idea that hypercholesteremic hyperlipemia is the principal initiating factor in most human athero-arteriosclerosis has little support. To be sure, lipid deposits may be demonstrated in early life but always in locations which also show intimal proliferative reactions independent of significant lipidosis. Under special conditions in later life, lipid deposits may occasionally be found in the absence of medial degeneration and intimal stromal reactions. These deposits, however, are seldom impressive and tend to occur in locations where intimal proliferation is ordinarily encountered even where there is no medial structure, as in cardiac valves. In other words, lipidosis of the intima is generally restricted to those parts of the arterial system which are susceptible to medial degeneration and reactive mesenchymal proliferation. This is equally true of the venous system if the pressure within the veins is sufficiently great. This is not true of the mesenchymal reactions. They often occur in parts of the vascular system which are ordinarily refractory to lipid accumulation and are commonly conspicuous without significant lipid accumulation, especially in arteries within the parenchyma of solid organs such as the brain, heart, kidneys, spleen, uterus and ovaries.

It is not correct to take the viewpoint that medial degeneration is always productive of mesenchymal reactions. For instance, in amyloid disease there may be profound medial degeneration without any change in the intima. The same statement may be made in connection with some instances of severe degenerative calcific disease, where at times mesenchymal reactions, if present at all, are minimal. This was so in the pulmonary venous system in these experiments. These facts, therefore, lead us to place emphasis, not upon medial degeneration *per se*, but upon those forms of medial degeneration which elicit regional mesenchymal reactions. The excitation of these reactions may be due to a number of factors, but except for rather uncommon infections or other inflammatory stimuli, the principal factor seems to be related to the development of mobile discontinuities of medial structure. The extent to which the discontinuities elicit mesenchymal reactions depends upon principles which determine the sequences in the evolution of such reactions in general.

Now, to what extent can the experimental observations assist in formulation of a consistent theory of the pathogenesis of the human disease? In the first place, the experiments showed that hyperlipemic hypercholesteremic atherosclerosis was characterized by severe intimal atheromatosis, but that this experimental disorder did not have the distribution of human athero-arteriosclerosis. Furthermore, the atheromatous lesions, as they underwent customary evolution into degenerated confluent masses of lipids and cholesterol, did not lead to calcific medial degeneration or elicit significant fibrogenic reactions. Indeed, the evidence was to the contrary. Calcific medial degeneration and intimal fibrogenic reactions were inhibited. The conclusion was that the hyperlipemic disorder is not a suitable reproduction of the usual human disease.

In the second place, the experiments disclosed that hypervitaminosis D was productive of a severe calcific-degenerative medial disease in many arterial systems. In the evolution of the disorder, lesions resembling those in the Mönckeberg type of human arteriosclerosis were reproduced in minute detail. Fibro-cellular thickening of the intima was a conspicuous feature. However, this experimental disease did not reproduce two important features of human athero-arteriosclerosis. It lacked the anatomic distribution. Also, it lacked intimal lipidosis unless there was a complicating viosterol-induced hypercholesteremia.

By combinations of dietary hyperlipemic hypercholesteremia and hypervitaminosis D, a more complicated vascular disorder was produced. At cholesteremic levels equal to or exceeding those common in the human adult, the anatomic distribution and the microscopic characteristics of this disorder varied with the relative influence of 4 rules of reaction. The first rule was that medial calcific degeneration in the absence of a local mesenchymal reaction was not conducive to lipid localization. The second rule was that medial calcific degeneration was conducive to lipid localization if there was an active mesenchymal reaction. The probability of localization depended principally upon temporal relations between the level of blood cholesterol, the duration and magnitude of the level, the natural lipidosis potential of the vascular segment and the extent to which this potential was increased by local mesenchymal activation. The third rule was that lipid localization without tissue necrosis was antagonistic to calcium accumulation through mechanisms of protection or accelerated resorption. The fourth rule was that lipid localization modified the usual normal sequences of vascularizing mesenchymal reactions. Proliferation of fibroblasts and endothelium of newly formed capillaries was retarded. The mesenchymal cells accumulated lipids. Intercellular fibrogenesis was retarded, and the fibrils which were formed became hyalinized.

If these rules derived from animal experiments are applicable to man, several predictions about human athero-arteriosclerosis can be made as follows:

(1) Degenerative medial arterial disease with or without calcification and irrespective of pathogenesis will in the absence of mesenchymal activation lead to elongation, dilatation and tortuosity of vessels.

(2) Degenerative medial arterial disease, irrespective of etiology, will as a rule elicit mural mesenchymal reactions. The characteristics of these reactions will be determined by conditions which ordinarily regulate mesenchymal activation, proliferation, differentiation and organization. The successive stages and the final result will be a reflection of this regulation so that a broad variable spectrum of mesenchymal medial and intimal reactions may be expected. In general, these will lead to elongation and tortuosity of vessels with variable decrease or increase in caliber.

(3) Ordinarily, medial degenerative arterial disease and the secondary mesenchymal reactions will not be appreciably modified by serum cholesterol levels below 150 mg. per hundred cc., but modifications may be expected at serum cholesterol levels of about 150 to 250 mg., irrespective of the cause of the elevation. The modifications will be minimal if the mesenchymal reaction has matured and activation subsided prior to attainment of the indicated levels. The modifications will be maximal if the cholesterol levels near the higher range are rising rather than falling and if they not only precede but also accompany the period of maximal mesenchymal activation. Lipids will localize only in the proliferating intima of those arterial segments with the highest natural lipidosis potential. The general result will be fibro-xanthomatous hyaline plaques in the intima of the vessels, with the most conspicuous changes in the proximal aorta and the proximal segments of its large branches.

(4) When serum cholesterol levels are about 250 to 350 mg., the same rules apply. In general, however, lipid accumulations in the intima will have a more widespread distribution because the lipidosis potential of more arterial segments will be increased, not only by local mesenchymal activation, but also by the repletion of other segments having still higher potentials. Under optimal conditions and especially with vascularized mesenchymal activation in the degenerated media, lipids may accumulate in the media of these naturally more resistant arterial segments. The usual result will be retardation of mesenchymal proliferation as the lipids accumulate and a conversion of the proliferating mesenchyme to a hyaline degenerated structure with a tendency for calcium to accumulate in the entrapped xanthomatous areas in the hyalinized mesenchyme. Under optimal conditions for their development, these

changes may become conspicuous in a few weeks, not necessarily requiring years for formation and involutive degeneration as generally believed. Actually, when cycles of optimal and suboptimal conditions of sufficient duration occur, intimal laminar arrangements of xanthomatous, fibro-xanthomatous, fibrous and fibro-hyaline mesenchyme may be expected to mark the duration and magnitude of the corresponding responsible conditions.

(5) The lipidosis potential of the normal arterial wall is so low that no significant intimal plaques will form at serum cholesterol levels below 350 mg. Even at levels as high as 500 mg., seldom found in man, the xanthomatous intimal plaques will be restricted principally to the arterial segments of highest potential, ordinarily located in the proximal aorta and the proximal parts of its major branches. At this cholesterol level, the lipidosis potential of normal arteries elsewhere will be less than the potential in many parts of the reticulo-endothelial system so that there will be a tendency toward lipidosis of this system rather than of distal arteries.

(6) Any process of mesenchymal activation independent of medial degeneration may lead to intimal lesions similar to those described as secondary to medial degeneration. Hence, under appropriate conditions the reparative sequences in adherent thrombi or those occurring in the course of endarteritis, mesarteritis or periarteritis may result in fibrous, fibro-xanthomatous or xanthomatous intimal lesions. Even the "normal" activation due to arterial growth secondary to increased circulatory demand or the "normal" activation due to arterial atrophy secondary to the reduction in circulatory demand may be a source of adequate intimal mesenchymal activation. In each instance the relation between the level of cholesterol in the blood and the local lipidosis potential will determine the xanthomatous evolution of the mesenchymal reactions.

(7) Conditions other than the most severe forms of intimal and medial disease are required to induce occlusive intra-arterial thrombosis. Among these conditions the presence of vascularized mesenchyme in the degenerated intima, an event which never occurred in these experiments, would seem to be the most important single local contributing factor in the human arterial wall itself.

It is believed, therefore, that a further evaluation of the proposed theory must await extension of 3 lines of research. The first should be concerned with the pathogenesis of calcific medial degeneration of arterial systems. The second should establish the limit of hyperlipemia or hypercholesteremia below which no significant lipid deposits will occur in the intima of critically important arterial segments, irrespective of the magnitude of calcific medial degeneration and associated mesen-

chymal reactions. The third should establish the nature of disturbances which, in the presence of athero-arteriosclerotic changes, lead to the development of occlusive arterial thrombosis despite normal or reduced clotting properties of the circulating blood.

Summary

Three types of arterial disease were produced in rabbits. One, characterized principally by intimal lipidosis, was produced by hypercholesteremic hyperlipemia. Another, characterized principally by medial calcific degeneration with secondary mesenchymal reactions, was produced by hypervitaminosis D. Neither disease resembled human atherosclerosis. The third, produced by permutations of the two pathogenetic mechanisms, was characterized by a broad spectrum of athero-arteriosclerotic changes closely resembling those commonly found in man. These changes reflected synergism and antagonism of the two mechanisms in preformed and activated arterial mesenchyme. The principal synergism was the tendency for rapid lipid localization to occur in arterial mesenchyme activated by medial calcific degeneration and for this localization to occur at serum cholesterol levels not necessarily exceeding those considered as normal for man. The rate and degree of lipid localization depended on several factors. Within a given effective range of serum cholesterol levels, the localization was most conspicuous in arterial segments having the highest natural lipidosis potential when the cholesterol level was rising and near its maximum during the period of greatest mesenchymal activation. Within a given effective range of serum cholesterol levels, lipid localization was least in arterial segments having the lowest natural lipidosis potential when the serum cholesterol level was falling near its minimum and when the mesenchymal activation had subsided.

Also, antagonisms between the two pathogenetic mechanisms were reflected in the reactions of activated mesenchyme as lipids were localized. The localization not only retarded the proliferation of fibroblasts, histiocytes and endothelium of newly formed capillaries but also converted them to "foam cells." As this occurred, fibrogenesis was inhibited and the fibrils which formed acquired an abnormal hyaline appearance. On the other hand, lipid localization had a protective action, for it retarded the progress of medial calcific degeneration and accelerated the local resorption of calcium already deposited, resulting in an increased tolerance of the hypercholesteremic animal to viosterol. But this tolerance was not wholly beneficial because of two curious effects. The first was the tendency for lipids to localize in sites having a lower lipidosis potential if the sites of higher potential were occupied either with lipids

or mature resistant proliferated mesenchyme. The second was the tendency for calcium to localize in sites having a lower calcific-degeneration potential if the sites having higher potentials were occupied by lipids or mature, newly formed mesenchyme. These tendencies led to the occurrence of complex disease in distal arterial systems normally spared at tolerable levels of hypercholesteremia or hypervitaminosis D.

These observations may assist in a better understanding of human athero-arteriosclerosis. It seems likely that most people with common serum cholesterol levels would be spared serious disease if the factors responsible for calcific medial degeneration could be controlled. While it is not likely that hypervitaminosis D is a factor in this degeneration, mechanisms similar to those activated by hypervitaminosis D may be important. It was clear that severe experimental changes in arterial walls, generally regarded as favoring thrombosis, proved to be unfavorable. Among all changes assumed to favor thrombosis in man, the only one unrecognized in these experiments was vascularization of degenerated intimal plaques, and this failed to occur even in the presence of rich vascularization of the subjacent media. The variable susceptibility of arterial segments even to the point of absolute resistance to the two diseases, singly or in combination, was most impressive. This variation was empirically defined in terms of lipidosis and calcific-degeneration potentials for which neither structure nor function of arteries provided an explanation. Whatever the explanation for these variable susceptibilities or potentials may be, there is no doubt that they also exist in man and govern the pattern of distribution of human athero-arteriosclerosis.

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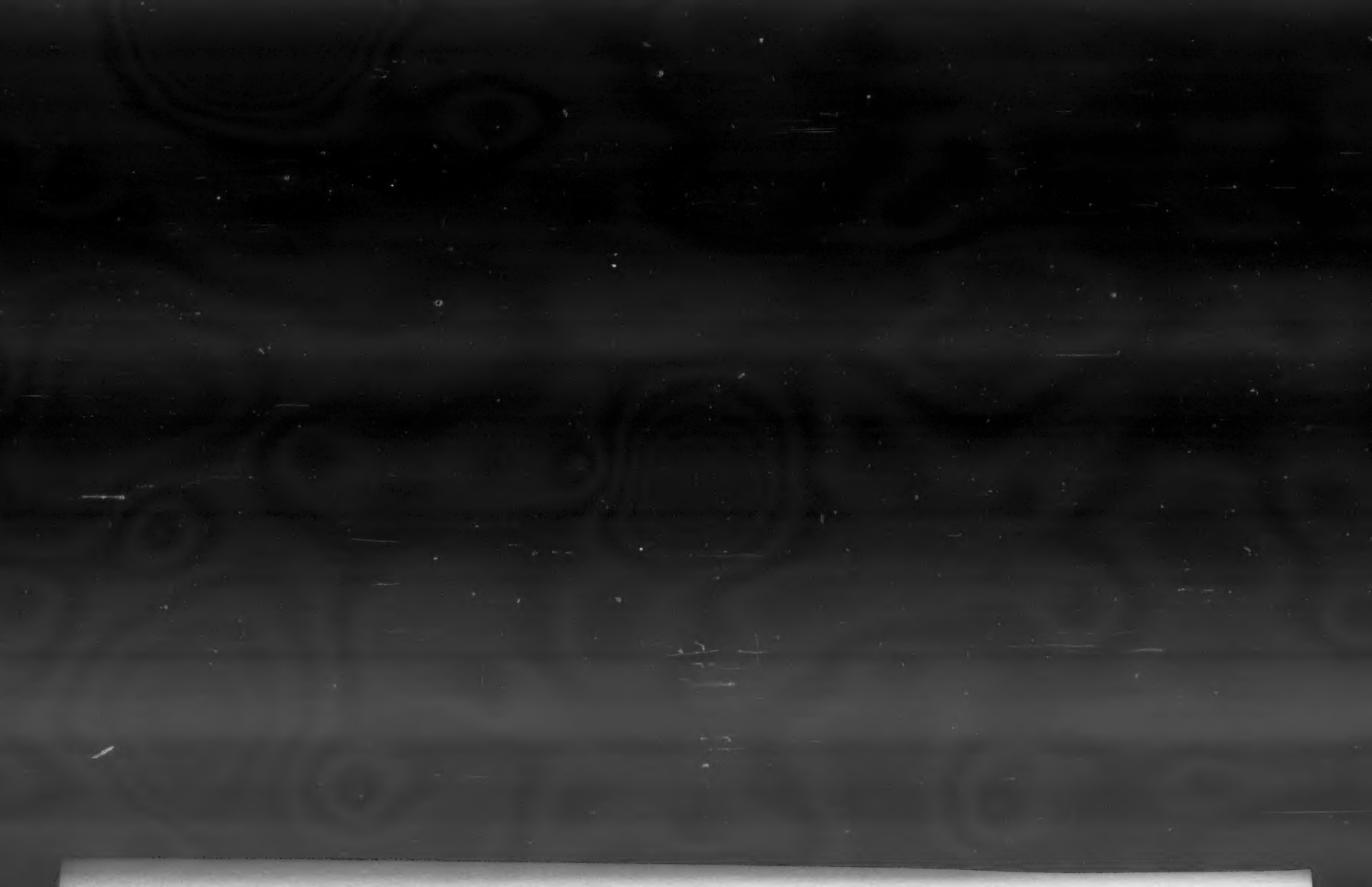
[*Illustrations follow*]

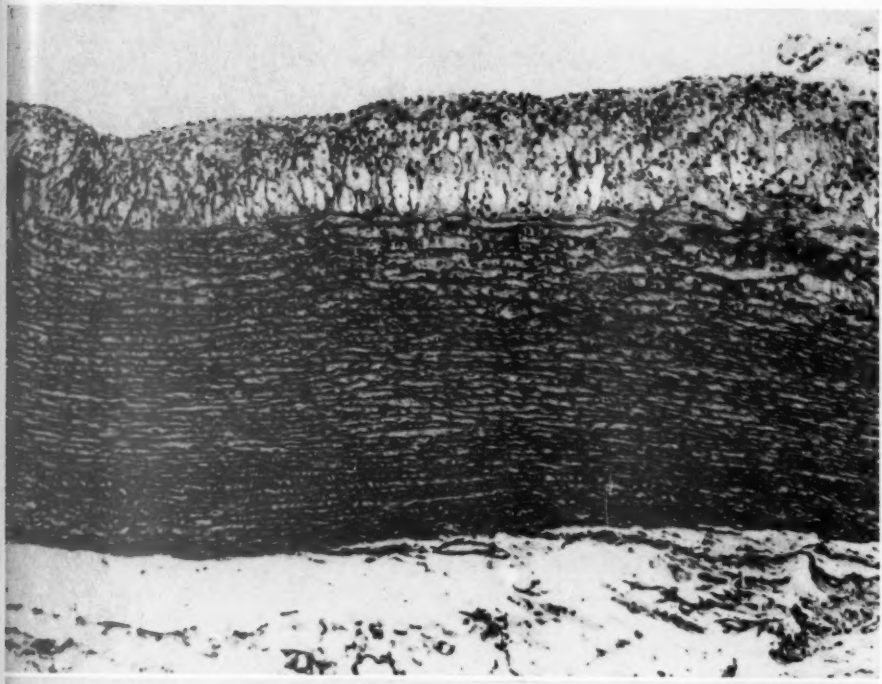
Legends for Figures

All photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. A cross section of the thoracic aorta in a rabbit given a 1.5 to 10 per cent cholesterol diet (caloric) for 14 weeks. The serum cholesterol gradually increased to a maximum of 2,100 mg. per hundred cc. There is a thick intima composed almost entirely of mesenchymal cells, laden with lipids. This proliferative reaction is not accompanied by significant formation of ground substance, collagen or elastic tissue. The media is well preserved except for a scattering of interstitial foam cells and a generalized increase of lipids in smooth muscle cells and fibrocytes. × 60.

Fig. 2. A cross section of the abdominal aorta in a rabbit which was on the following successive regimes: first, 10 per cent cholesterol diet (caloric) for 4 weeks; second, 1.5 per cent cholesterol diet (caloric) for 4 weeks; third, a normal diet for 22 weeks. Serum cholesterol rose to 580 mg. in 10 weeks and fell to 80 mg. at the end of the experiment. This represents a common end stage of involution of a typical xanthomatous plaque. The moundlike intimal plaque was initially composed of "foam cells." As these cells, engorged with lipids, degenerated *in situ*, a loose, reticular, relatively acellular structure remained. In the interstices there are masses of crystal clefts presumably representing cholesterol, its esters and saturated fatty acids, which accumulated as the "foam cells" degenerated. Other lipids demonstrable in special stains of fresh frozen sections, not treated with fat solvents, are also present in lesions of this kind. All xanthomatous plaques tend to undergo this involution regression without significant formation of collagenous or elastic fibers, even in the presence of persistent high serum cholesterol levels. Note the retention of an essentially normal media in this instance. In instances when the media is xanthomatous, the involutional changes are similar to those shown here in the intima. × 160.





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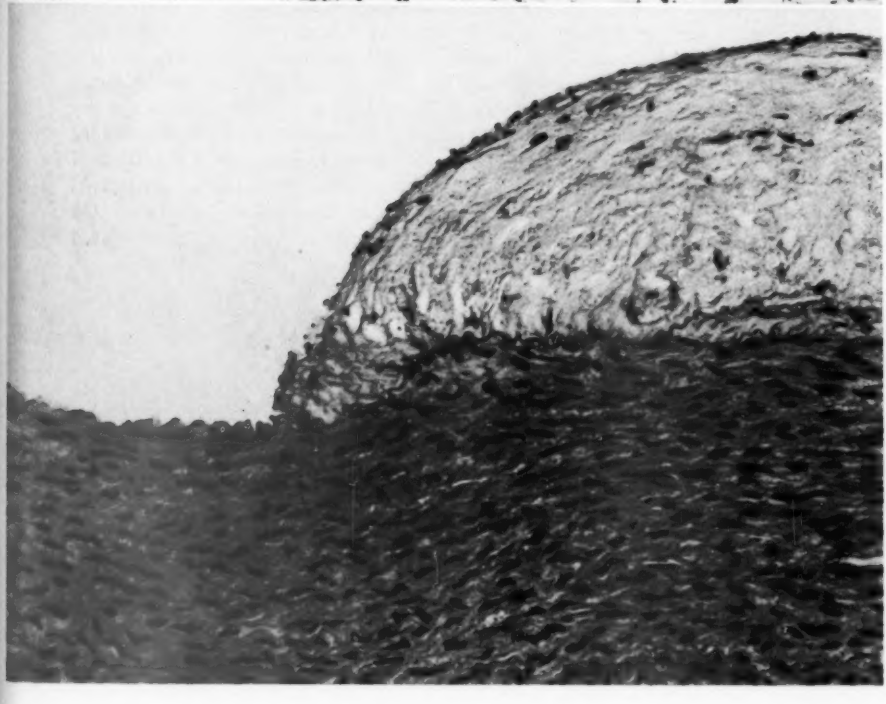

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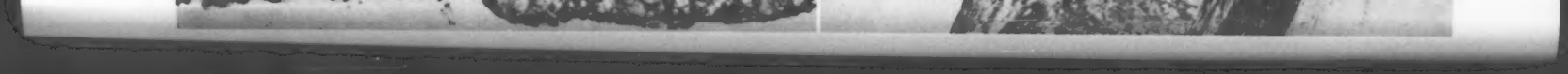
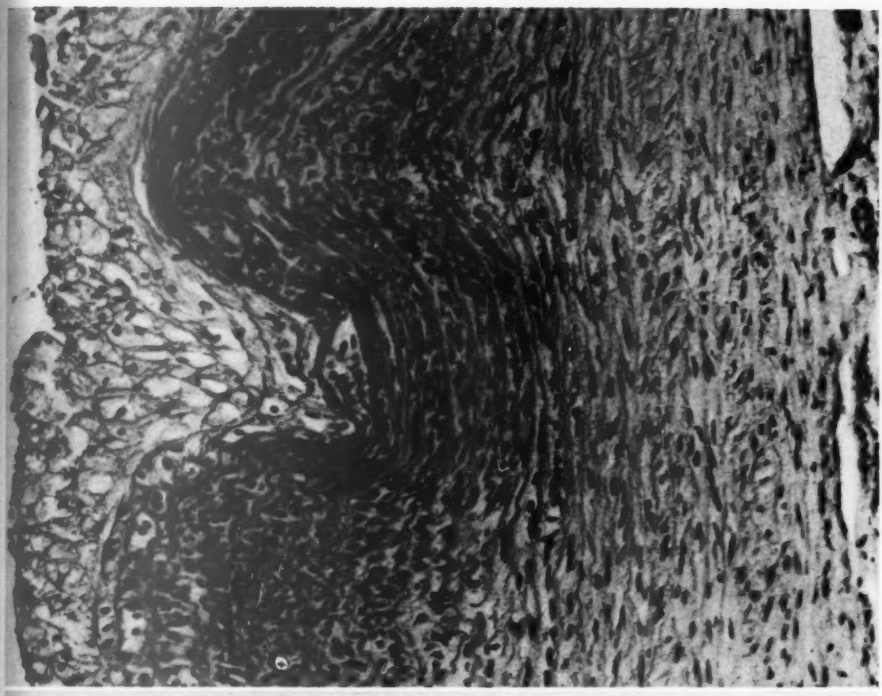
FIG. 3. A cross section of the thoracic aorta in a rabbit given 10 per cent cholesterol diet (caloric) and 300,000 units of viosterol each week for 5 weeks with the serum cholesterol rising to 2,680 mg. The elastic lamellas of the inner third of the media are stained heavily with hematoxylin, indicating calcification. Throughout this area there is a heavy infiltration of "foam cells" which is also responsible for the loose-textured appearance of the overlying xanthomatous intimal plaque. This illustrates two points: first, that the usual active fibrocellular mesenchymal reaction in the intima and media, secondary to calcific degeneration, is converted into a xanthomatous noncollagenous reaction in the presence of an appropriate hypercholesteremic lipemia; second, the inhibition of progressive calcification of the media and acceleration of resorption of calcium, already deposited by the xanthomatous localization in activated intimal and medial mesenchyme. × 140.

FIG. 4. Arch of the aorta in a rabbit maintained on the following successive regimes: first, 10 per cent cholesterol diet (caloric) for 6 weeks; second, 4 weeks of a 1.5 per cent cholesterol diet (caloric) with serum cholesterol rising to 2,970 mg.; third, 200,000 units of viosterol every fourth week for 41 weeks with serum cholesterol gradually falling to 160 mg. The thickened intima consists of a poorly cellular mass of degenerated atheromatous material with large, darkly stained deposits of calcium. The subjacent media shows a moderate accumulation of lipids in cells between the elastic lamellas. This demonstrates that a thick intimal xanthomatous plaque prevents medial calcific degeneration even when the viosterol regime is sufficiently severe and prolonged to have regularly produced excessive calcification in the full thickness of the media. × 150.





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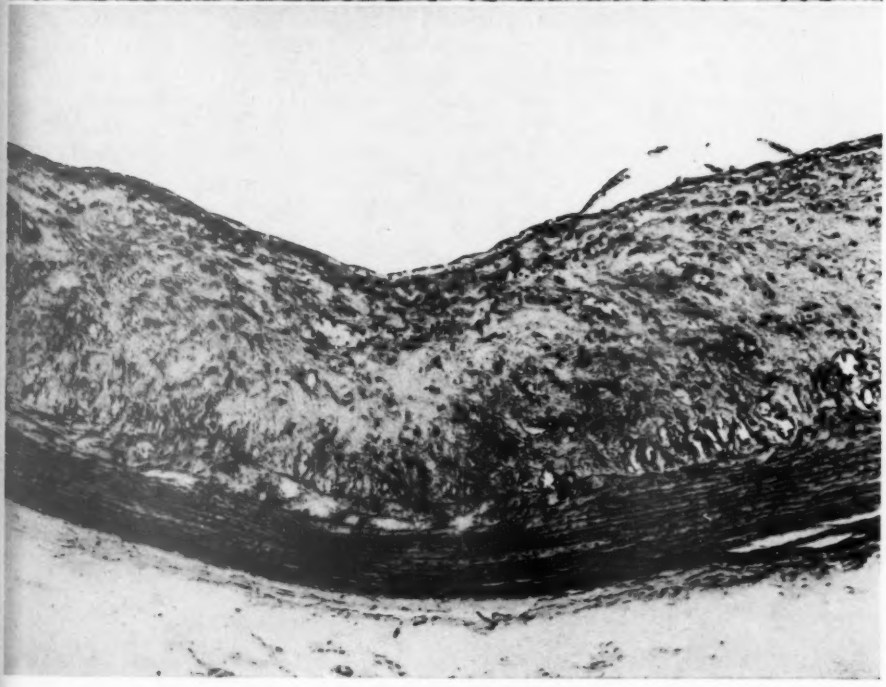

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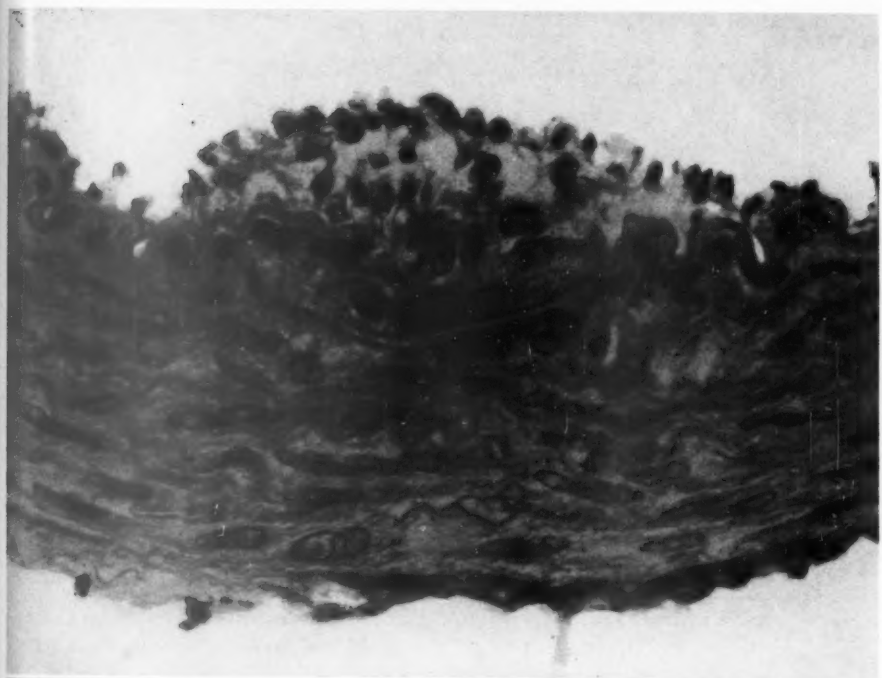
FIG. 5. A cross section of a carotid artery in a rabbit given 100,000 units of viosterol twice weekly for 5 weeks and a daily dose of Inversine® (7.5 to 5 mg. per kg. of body weight). There was no dietary supplement of cholesterol. Serum cholesterol values rose to a maximum of 180 mg. This shows 4 early simultaneous reactions: first, there is a homogeneous deposit of calcium just beneath the internal elastic membrane; second, there is discontinuity and fraying of the internal elastic membrane; third, there is a local mesenchymal proliferation, more conspicuous in the intima, where a tiny mound of mesenchyme has formed and acquired an endothelial covering; fourth, vacuolated lipid-laden cells are present in the proliferating intima. × 420.

FIG. 6. A cross section of a femoral artery in a rabbit kept on the following successive regimes: first, 200,000 units of viosterol each week for 4 weeks; second, 10 per cent cholesterol diet (caloric) for 4 weeks; third, 200,000 units of viosterol each week for 4 weeks; fourth, 10 per cent cholesterol diet (caloric) for 4 weeks. The total duration was 16 weeks, and the maximum serum cholesterol value was 680 mg. There is discontinuity of the internal elastic membrane over the darkly stained focus of calcific medial degeneration. Overlying the area of medial degenerative disease, there is a sharply localized mound of proliferating intima composed mostly of lipid-laden "foam cells." Plaques of this kind stain intensely with special stains for lipids. In this animal there were no xanthomatous intimal plaques anywhere except over areas of medial calcific degenerative disease. × 320.



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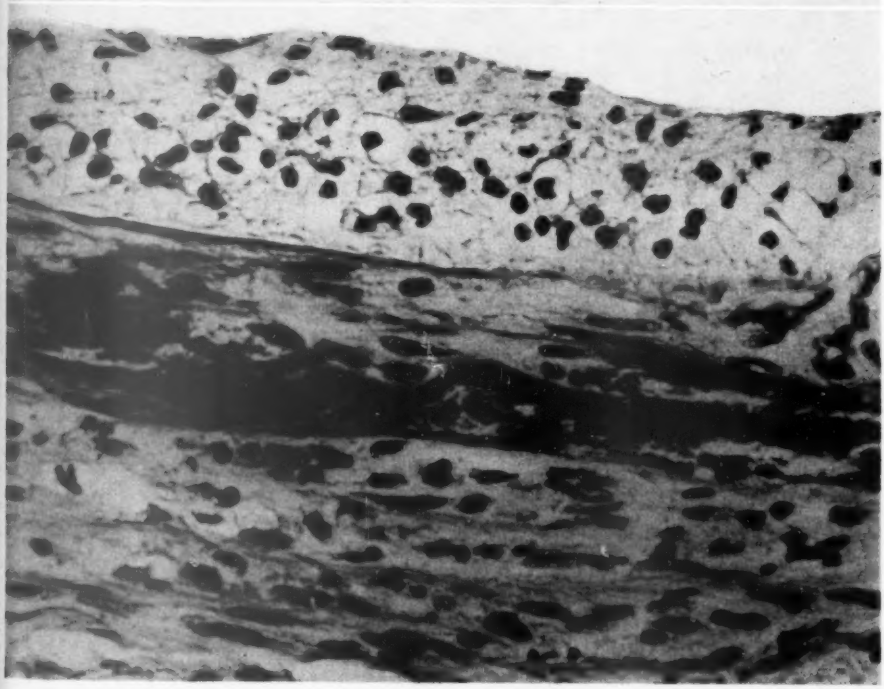

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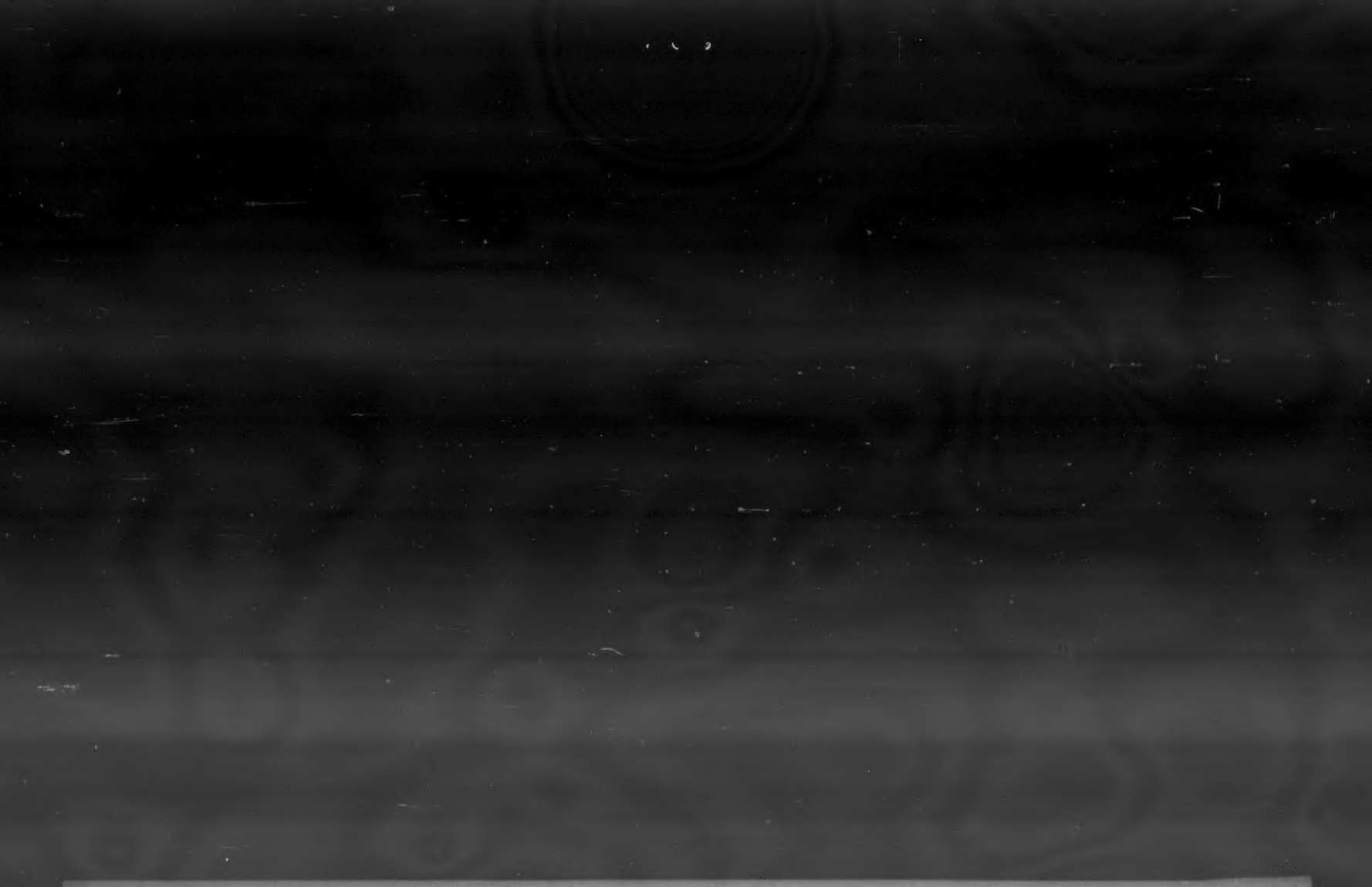
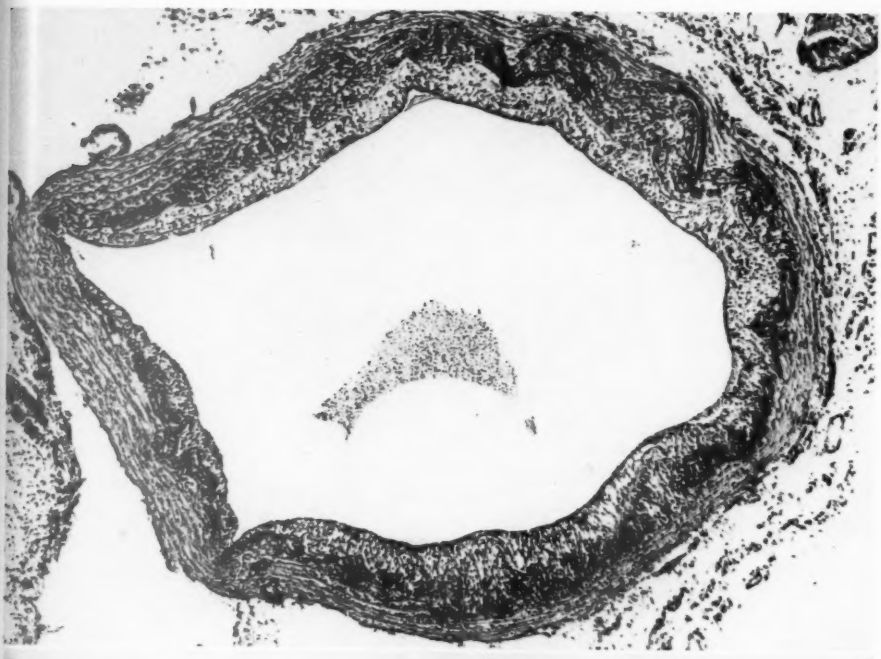
FIG. 7. A cross section of a carotid artery in a rabbit kept on the following successive regimes: first, 6 weeks with 100,000 units of viosterol twice each week; second, 4 weeks on a 10 per cent cholesterol diet (caloric) with serum cholesterol rising to 430 mg.; third, 2 weeks on a normal diet without viosterol. The darkly stained band represents the location of calcium deposits, principally associated with the elastic lamellas and interlamellar structure of the inner half of the media. External to this is normal media. Internal to this is a broad layer of thickened intima composed of immature fibroblasts and closely packed xanthomatous cells undergoing disintegration. This represents fibro-cellular mesenchyme. actively proliferating in response to medial calcific degeneration, being converted into xanthomatous intimal mesenchyme. This occurred during 6 weeks of increasing serum cholesterol levels to a modest maximum of 430 mg. An optimal coincidence of calcific medial degeneration, maximum activity of secondary intimal proliferation and rapidly increasing hypercholesteremic hyperlipemia is a requirement for this pronounced effect. × 80.

FIG. 8 A cross section of a femoral artery in a rabbit kept on the following successive regimes: first, 12 weeks with 200,000 units of viosterol every fourth week; second, 24 weeks on a 1.5 per cent cholesterol diet (caloric) with serum cholesterol values rising to a maximum of 4,160 mg.; third, 12 weeks of a normal regime with serum cholesterol falling to 210 mg. This illustrates certain sequences. During the first 12 weeks medial calcific degeneration occurred in focal areas extending from internal elastic membrane to adventitia. This elicited proliferation of intimal and medial mesenchyme which attracted atheromatous localization during the next few weeks, characterized by a great rise in blood cholesterol and other lipids. The "foam cells" initially infiltrating the fibrocellular proliferated intima adjacent to the internal elastic membrane degenerated, leaving a fibro-hyaline structure with extracellular crystalline deposits represented here as pale, blotchy, unstained areas. The "foam cells" in the media persisted, marking the area formerly occupied by calcium, a narrow, darkly stained rim of which still remains. In the last few weeks, as normal blood cholesterol levels were approached, the continuous reparative intimal reaction acquired a healthy fibro-cellular structure forming a new lining to the lumen of the artery. Vascular degenerative and reparative changes of this kind occurred only in prolonged experiments and, as might be expected, more closely resembled the chronic lesions encountered in human disease. × 150.





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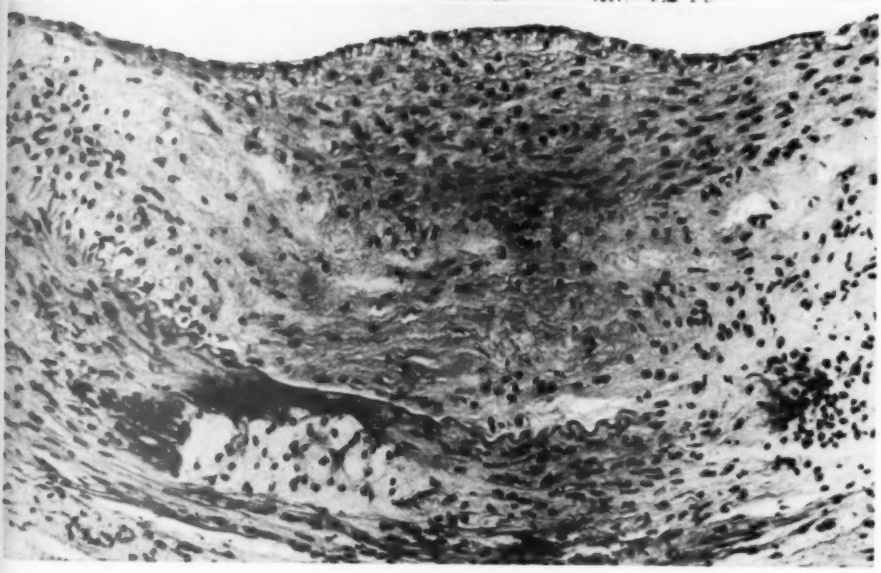

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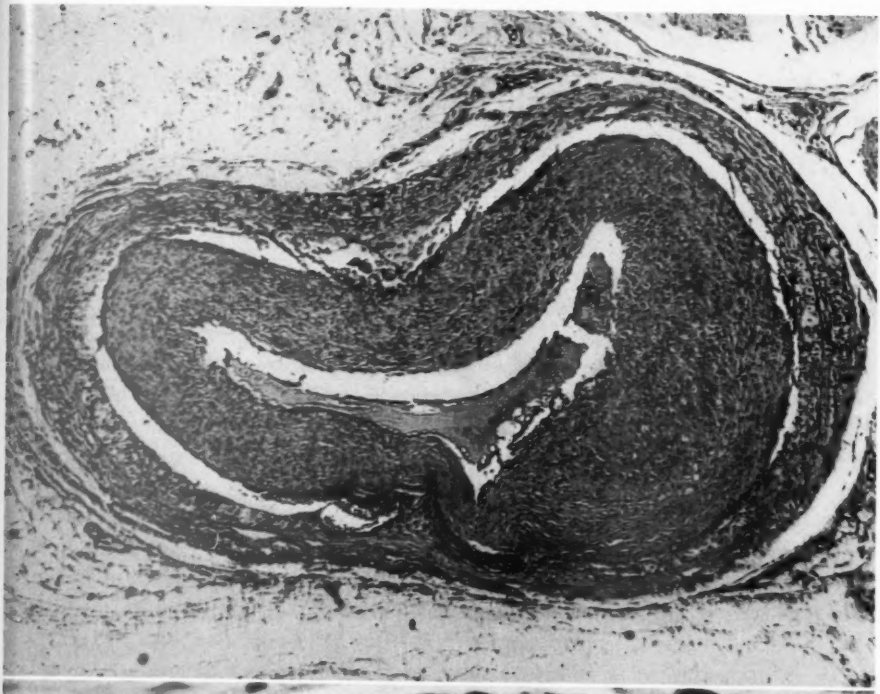
FIG. 9. A femoral artery in a rabbit kept on the following successive regimes: first, 200,000 units of viosterol every fourth week for 12 weeks; second, 200,000 units of viosterol every fourth week and a 1.5 per cent cholesterol diet (caloric) for 48 weeks. The serum cholesterol values reached a plateau at 2,000 to 2,500 mg. This represents one of several end results of combining the two pathogenetic mechanisms for production of arterial disease. During the initial period of viosterol administration, the full thickness of the media underwent calcific degeneration. This excited an intimal proliferative reaction and a penetration of the calcified media by vascularized mesenchyme originating in the adventitia. Intimal proliferation was complete and mature before hypercholesteremia developed. It was thereby resistant to xanthomatous transformation and accumulation, remaining densely fibro-cellular with a few lipid deposits. The mesenchymal activity in the media, however, was at its peak during the stage of increasing hypercholesteremia. Xanthomatous localization occurred here and stimulated calcium resorptive mechanisms so that the media disappeared and, as shown, the adventitia and thickened intima were all that remained. There is considerable resemblance between these changes and those often found in the femoral and tibial arteries in man. × 40.

FIG. 10. A small artery in the submucosa of the stomach in a rabbit kept on the following successive regimes: first, 200,000 units of viosterol every fourth week for 12 weeks; second, 200,000 units of viosterol every fourth week for 36 weeks and a 1.5 per cent cholesterol diet (caloric). Serum cholesterol values rose to a plateau of 1,200 to 1,500 mg. This illustrates an end result of a chronic viosterol-hypercholesteremic regime lasting 48 weeks. The once heavily calcified media of this artery has undergone internal and external changes. The pale, slightly porous homogeneous hyaline external tissue represents the degenerated medial structure from which calcium has been absorbed. Often, the hyaline matrix of slowly demineralized, degenerated media stained brightly with eosin and thus resembled the hyaline medial matrix so common in human arteriosclerosis. The intermediate ring of darkly stained tissue is the remnant of the media which has retained some calcium. This is mottled and partly resorbed, especially on its inner aspect. Here it lies adjacent to a thick layer of intima composed mostly of vacuolated lipid-laden cells surrounding and encroaching upon the much narrowed lumen. In different animals, there were many complex variations in this local interplay of reversible calcium deposition, variable mesenchymal activity and reversible xanthomatous accumulation. × 480.



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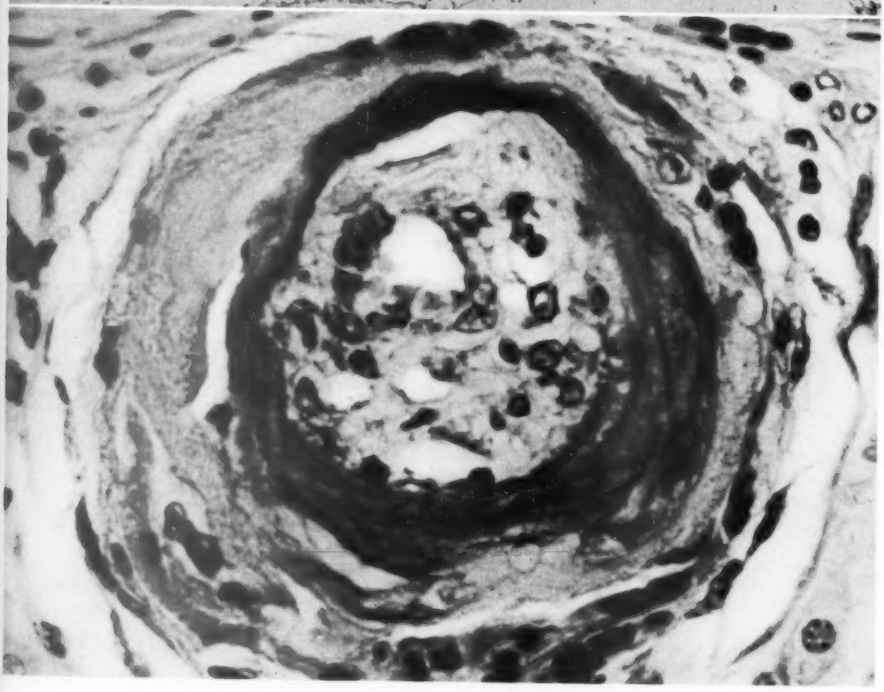

10

THE EFFECTS OF AMINOPTERIN ON GUINEA PIG TUBERCULOSIS

ROBERT W. PRICHARD, M.D., AND DONALD M. HAYES, M.D.

From the Department of Pathology, Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N.C.

This paper discusses the effect of a folic acid antagonist, aminopterin (4-aminopteroyl glutamic acid), on several manifestations of tuberculosis in the guinea pig. The study was originally undertaken to aid in the evaluation of an instance of miliary tuberculosis discovered incidentally at necropsy examination in a child with acute leukemia; the patient had received aminopterin therapy. The possibility that a leukemoid reaction to tuberculosis had been misinterpreted as leukemia was considered. However, there was widespread evidence of leukemic involvement, and the possibility of a leukemoid reaction was dismissed as unlikely. In addition, the histologic appearance of the tubercles indicated that they were of much more recent origin than the 6-month duration of the child's illness. The known cytotoxicity of aminopterin raised the possibility that the drug had interfered with the evolution of the tubercles. An animal experiment was carried out in order to make an objective determination of this phenomenon.

MATERIAL AND METHODS

A 6-week investigation of the interaction between aminopterin and tuberculosis was planned. Young guinea pigs, the H37RV strain of tubercle bacilli, and aminopterin were utilized. There were 3 experimental groups of 11 guinea pigs each; these were roughly equal in sex distribution. Group I was inoculated with tubercle bacilli alone. Group II received inoculation with tubercle bacilli followed by the administration of aminopterin begun at the appearance of cutaneous tuberculin hypersensitivity. Group III was inoculated with tubercle bacilli, and aminopterin administration was begun simultaneously.

Additional groups of 4 guinea pigs each were kept as environmental and aminopterin controls.

The guinea pigs weighed 100 to 200 gm. and, for the experimental groups, were obtained in litters of 3, one animal being placed in each group. They were caged separately, fed Purina Chow and given water *ad libitum*. Body weights were recorded at the beginning of the experiment and at weekly intervals thereafter.

Tubercle bacilli of the H37RV strain were suspended in 0.5 ml. of physiologic saline and administered by subcutaneous injection of approximately 450×10^6 organisms into the right groin. The suspension was evaluated turbidimetrically, by making it equivalent to the #3 standard of the McFarland nephelometer. The organisms in

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this volume of inoculum weigh roughly 1 mg. wet (10 ml. of inoculum contained 19.7 mg. of organisms). This weight contains the numbers of organisms cited above.[1]

Aminopterin administration was carried out by the subcutaneous injection of 2 μg. per gm. of body weight per day. Groups II and III and the drug controls received the substance.

Tuberculin testing was carried out on all animals at the start of the experiment and at 7-day intervals during the succeeding 6 weeks. The technique of giving and interpreting the tests was that of Holm and Lind,[2] using intradermal inoculation of 0.1 ml. of 1/1000 old tuberculin.[3]

At the end of the 6-week period, all living animals were sacrificed. These animals and those who died during the course of the experiment were necropsied. Gross counts of tubercles in various organs were made. Specimens were prepared for microscopic examination from the liver, spleen, regional lymph nodes and bone marrow. Semiquantitative evaluation of various factors was made on the basis of slight, moderate and marked.

Results

Mortality (Table I)

Of the 11 animals who received only tubercle bacilli, 3 died late in the experiment. There were 2 deaths among the animals who were to receive aminopterin at the time of cutaneous tuberculin hypersensitivity before that hypersensitivity appeared, so they are, in effect, tuberculous

Table I

DAY OF DEATH

	Days from beginning of experiment					
	7	14	21	28	35	42
Group I				1	2	
Group II	1		1	1	2	1
Group III	1	3	2	2	1	

control animals. They died at 7 and 35 days. Of the 9 animals who became tuberculin positive and received aminopterin, 4 died during the last 2 weeks of the experiment. The animals receiving aminopterin early (group III) had the highest mortality, with only 2 animals surviving for 6 weeks. Most of the deaths in this group were between the 14th and 28th days. Two of the 4 aminopterin controls died at 28 and 35 days. Weight gain in the aminopterin-treated animals was less than that of the tuberculous or environmental controls.

Cutaneous Tuberculin Reaction (Table II)

All of the animals in groups I and II developed a positive tuberculin reaction with the exception of the 2 animals in group II who died. None of the animals in group III became tuberculin positive although most of them lived long enough to have become positive when compared with groups I and II. This is demonstrated by applying to group III the rate

at which animals in groups I and II became tuberculin positive. On the 21st day of the experiment 13 of 21 surviving guinea pigs in groups I and II were tuberculin positive. Applying this proportion to the 5 surviving animals in group III, one would have expected 3 to be tuberculin

TABLE II

CUTANEOUS TUBERCULIN SENSITIVITY

	Days from beginning of experiment					
	7	14	21	28	35	42*
Group I		3†	4	2	1	1
Group II	*1*	3	3	2	*1*	1
Group III	*1*	*4*	*3*		*1*	*2*

* Sacrifice.

† Arabic numerals denote animals which became tuberculin positive and are placed on the day positivity developed. Numbers in italics denote animals which died and are placed on the day of death.

positive; none were. Furthermore, by the end of the experiment all surviving animals of the first 2 groups were tuberculin positive, while the 2 survivors of group III were still tuberculin negative.

One of the environmental control animals became tuberculin positive at 28 days. When necropsied at the end of 6 weeks there was neither gross nor microscopic evidence of tuberculosis. The reason for the sensitization is unknown.

Morphologic Observations (Table III)

Postmortem autolysis rendered several animals in groups II and III inadequate for study. The animals receiving only tubercle bacilli had the usual features of tuberculosis in the guinea pig. There were extensive lesions in regional lymph nodes, spleen and liver. The lesions were

TABLE III

PATHOLOGIC ALTERATIONS

	Adequate study material	Degree of tuberculous involvement	Degree of caseation necrosis	No. of acid-fast bacilli
Group I	All 11	Marked	Moderate	Few
Group II	8 of 11	Moderate	Slight	Moderate
Group III	7 of 11	Moderate	Absent	Many

characterized by a moderate amount of caseation necrosis and contained small numbers of tubercle bacilli. The animals that received tubercle bacilli and aminopterin from the start of the experiment had a slightly smaller degree of gross organ involvement. There was no caseation

necrosis, and the lesions contained large numbers of tubercle bacilli. The animals in group II, who received tubercle bacilli and aminopterin after the development of a positive tuberculin test, exhibited features midway between those in groups I and III (Figs. 1 to 6).

Discussion

The experimental results, in relation to the patient precipitating this inquiry, indicate that tuberculosis occurring in a person receiving aminopterin could possibly produce a somewhat different morphologic pattern than would be observed in an untreated individual. There are so many variables that the extent of this alteration would be unpredictable in a given human host. It appears, however, that one could place less faith in pathologic estimates of the duration of the tuberculous process in a patient under treatment with folic acid antagonists.

More directly, aminopterin administration at toxic levels appears to prevent the appearance of cutaneous tuberculin hypersensitivity and of caseation necrosis. It also appears that host mortality and numbers of tubercle bacilli in lesions are increased. Since it is generally agreed that caseation necrosis does not develop in tuberculous granulomas until hypersensitivity appears, these two observations are consistent with each other. The lack of caseation necrosis does not stem from an effect of aminopterin upon the tubercle bacillus itself, since the animals so treated exhibited large numbers of bacilli in their lesions, a situation which would usually be associated with extensive caseation. There is nothing in the findings of this experiment to suggest the fashion in which aminopterin produces its effects.

We have found no previous observations on the effect of aminopterin in tuberculosis, human or experimental. Previous work in two general fields deserves consideration: the effect of antimetabolites on various cells and on antibody formation; and the relationship between tuberculin hypersensitivity and caseation necrosis.

The Effects of Antimetabolites

The variety of cells affected by aminopterin is wide; there are especially striking effects on the bone marrow, lymphocytes and intestinal mucosa.[4] The stratified squamous epithelium of the skin is also affected.[5]

Wissler, Fitch, LaVia and Gunderson[6] have reviewed the reports of cells considered to be participants in antibody formation and presented their own observations in rats immunized with a single dose of particulate antigen (formolized *Salmonella typhi*, sheep erythrocytes or heat-killed Friedländer's bacilli). They described, in particular, a large

pyroninophilic cell which arises by mitotic division from a "primitive fixed reticular cell whose phagocytic powers seem to be limited, if they exist at all." They considered this to be the principal source of antibody. Total body x-irradiation, prolonged dietary protein deficiency and the "acute" administration of an amino acid antagonist which inhibits antibody formation all depressed the immune response and interfered with the proliferation and differentiation of this large antibody-forming cell.

Schwartz, Eisner and Dameshek [7] studied the effect of a purine antagonist (6-mercaptopurine) on the immune response of rabbits. A dose which suppressed the primary immune response to a soluble protein antigen (human serum albumin) only delayed the secondary response. The linear relationship between the amount of antagonist and the degree of antibody suppression led them to consider a first-order chemical reaction rather than cytotoxicity as the mechanism of action of this drug. Little, Oleson and Roesch [8] gave aminopterin, stilbestrol and testosterone, alone and in combination with pteroylglutamic acid, to chicks immunized with *Brucella abortus, Salmonella typhosa* and *Pasteurella multocida*. The antibody titers against these organisms were investigated before and after drug administration. Their results indicated that all of these drugs depressed antibody formation by pteroylglutamic acid antagonism, but the mode of action was unknown.

Haas, Stewart and Briggs [9] studied the effects of amethopterin (4-amino, 10-methyl pteroylglutamic acid), a folic acid antagonist closely related to aminopterin, on lymphocytic choriomeningitis virus infection in mice. Usually fatal doses of virus did not cause death in the treated animals although virus was recovered from them in quantities comparable to those procured from untreated control mice. Mice maintained on diets deficient in folic acid were also spared. Citrovorum factor eliminated the sparing effect of amethopterin. Lerner and Haas [10] examined microscopically the organs of mice dealt with in these experiments. The characteristic pathologic lesions of lymphocytic choriomeningitis were absent in 82 per cent (18 of 22 mice) of the animals in the acute stage of the disease, and from 33 per cent (4 of 12 mice) in the chronic stage. They interpreted this to indicate a delay rather than a suppression of the tissue response to the virus, and possibly as evidence of the development of some degree of immunity. Since a significant number of animals exhibited a tissue reaction of the usual type in response to the virus, the sparing effect of amethopterin was apparently not due to suppression of inflammation. However this drug produced its effect in mice, there would seem to be some similarity to the effects of aminopterin in guinea pig tuberculosis, since the host reaction was altered without apparent effects upon the disease-producing agent.

Cocchi [11] investigated the effect of aminopterin on vaccinia in rabbits. The cutaneous response to the vaccinia virus was not different from that in untreated control rabbits.

To summarize, it is known that aminopterin is toxic to the cells of the spleen, lymph nodes and bone marrow, the organs chiefly affected by tuberculosis in this experiment. In addition, the drug profoundly alters the precursors of exudative cells appearing in tuberculosis and, in all probability, the cells which produce antibody. The separation of the relative importance of these possibly different actions will require additional experimentation.

Relationships of Hypersensitivity to Caseation

Rothschild, Friedenwald and Bernstein [12] contrasted tuberculosis in the immune allergic guinea pig with that in the immune desensitized guinea pig, normal guinea pig, and in animals receiving injections of glycerine. Immunity was produced by infection with the avirulent R1 strain of tubercle bacilli. Desensitization was effected and maintained by the subcutaneous injection of large amounts of old tuberculin and purified protein derivative. The animals were all then inoculated with the H37RV tubercle bacillus. In the desensitized animals "in general, . . . the lesions were harder and showed less necrosis" than in the immune allergic animals. Immunity, as reflected in mortality, course, spread and severity of the disease, was not affected by the procedure, and desensitization was, possibly, even beneficial.

Follis [13] prevented the development of hypersensitivity to old tuberculin by administering that substance to guinea pigs who were infected with virulent tubercle bacilli. Their lesions showed "less caseation than those of controls" that develop hypersensitivity. Follis commented upon the emaciation of many of the animals receiving old tuberculin, an observation that was also made by Rothschild and co-workers.[12]

The action of aminopterin in suppressing caseation and cutaneous tuberculin hypersensitivity seems quite similar to that effected by desensitization. The more profound action of aminopterin may simply be related to its greater toxicity, but the possible effects of aminopterin on endothelium and other elements of the local reaction may also be important. The establishment of a dose-depression relationship may be helpful in further investigation.

A digression on the action of cortisone is worthy of mention at this point. In general, cortisone administration in experimental tuberculosis results in a greater degree of tuberculous involvement, with lesions exhibiting lessened caseation and increased numbers of tubercle bacilli.[15] There is evidence suggesting that alterations in connective tissue and

lessened reactivity of small blood vessels play an important part in this altered response.[14,15] The action of cortisone suggests the importance of nonimmunologic features in the bodily response to tuberculosis.

Summary

Guinea pigs infected with a virulent strain of tubercle bacilli did not develop hypersensitivity to old tuberculin when given aminopterin in toxic doses. This procedure abolished caseation in the tuberculous granulomas as well; there was no effect, other than a possible augmentation, on the numbers of tubercle bacilli in the lesions.

The action of aminopterin is superficially similar to that of desensitization by old tuberculin. Nothing is known of the mechanism of action of either modality.

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Drs. Sidney Farber, Israel Diamond and George Foley supported an unreported initial study similar to the present one, carried out by the senior author at the Children's Medical Center, Boston. No microscopic observations were made on this material.

Dr. Hayes's work was done during the tenure of a student fellowship of the National Foundation.

Dr. J. M. Ruegsegger of Lederle Laboratories supplied the aminopterin used in these studies.

LEGENDS FOR FIGURES

Except where indicated, photographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. A splenic granuloma in a group III animal (aminopterin-treated from start), 23 days after inoculation with tubercle bacilli. There is no caseation necrosis. × 225.

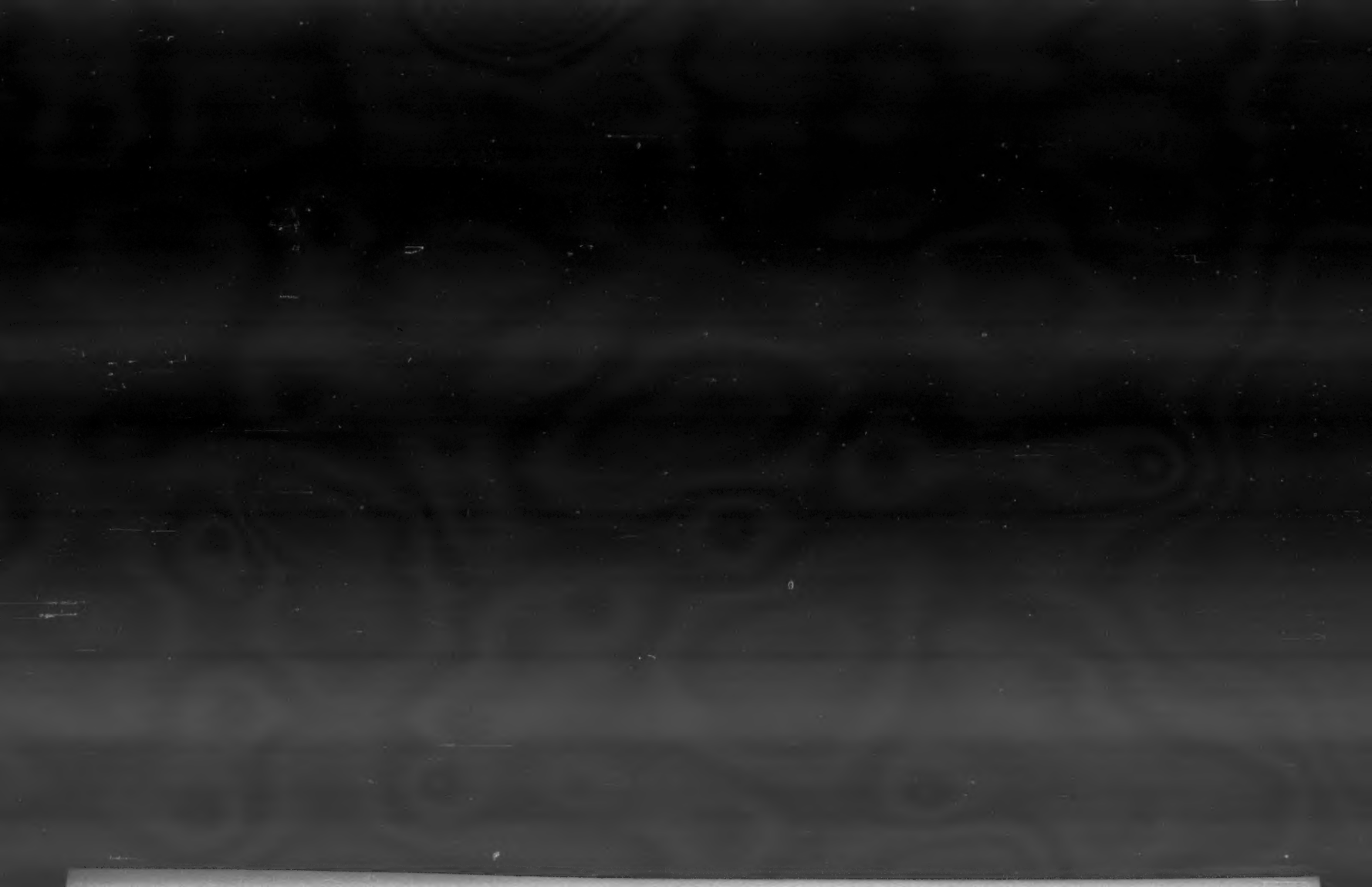
FIG. 2. The same tubercle shown in Figure 1, stained to demonstrate tubercle bacilli. Ziehl-Neelsen carbol fuchsin stain. × 480.

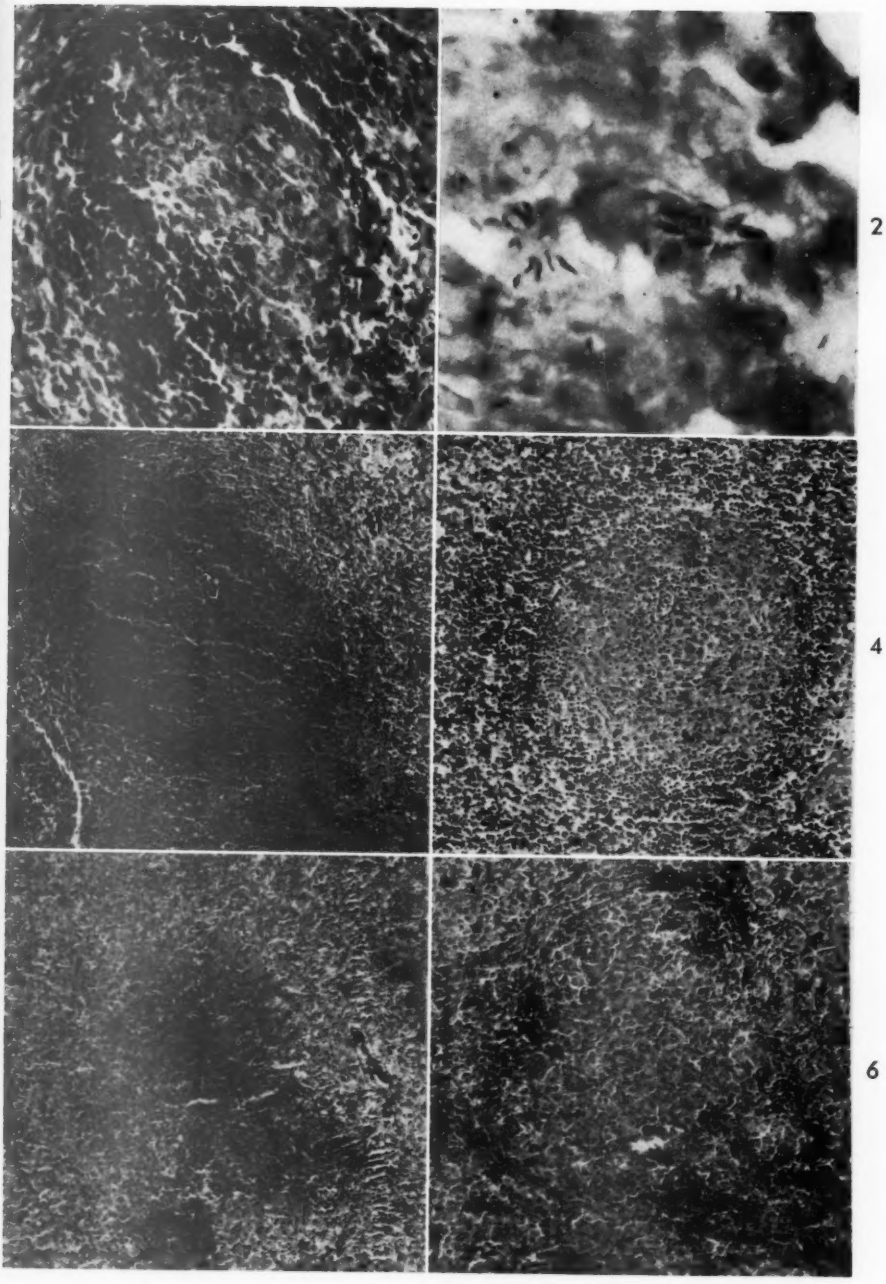
FIG. 3. A lymph node granuloma in a group I animal (inoculated only with tubercle bacilli) 28 days after inoculation. Caseation necrosis is marked. × 127.

FIG. 4. A splenic granuloma in a group III animal, 33 days after inoculation. There is no caseation necrosis. × 127.

FIG. 5. Lymph node granulomas in a group I animal sacrificed 42 days after inoculation with tubercle bacilli. Caseation necrosis is moderate. × 127.

FIG. 6. Lymph node granuloma in a group III animal sacrificed 42 days after inoculation with tubercle bacilli. There is no caseation necrosis. × 127.

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VITAMIN A DEFICIENCY IN THE GERM-FREE RAT

DAVID L. BEAVER, M.D.

From the Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

Deficiency of vitamin A in experimental animals is so regularly associated with infection[1,2] that the term "anti-infective vitamin" has often been applied.[3] It has been pointed out, however, that secondary bacterial invasion may actually be due to alterations in mucous membranes rather than to lowered resistance *per se*.[4] These mucosal changes were first emphasized by Wolbach and Howe,[5] who found cornification of the epithelium of various organs. Since that time "keratinizing metaplasia" has been accepted as a specific pathologic manifestation of avitaminosis A and the *sine qua non* of A deficiency in experimental animals.

In A-deficient conventional animals, the almost constant occurrence of infection obviates the necessity of postulating a specific cause of death. In the germ-free animal such is not the case. In fact, if it is true that simple epithelial alterations in the mucosa and ducts of nonvital organs are the only significant changes which occur, then the peculiar environment of the A-deficient germ-free rat should be conducive to long life and relatively good health. It was felt, therefore, that a study of vitamin A deficiency in the germ-free animal would not only provide an unclouded pathologic picture of tissue changes which have heretofore been obscured, but would also clarify the role of secondary infection in the pathogenesis of the disorder.

MATERIAL AND METHODS

Ten germ-free Lobund albino weanling rats, 7 females and 3 males, were transferred from a Reyniers holding tank to a Trexler type plastic isolator,[6] where they were housed in pairs in wire-bottom cages and maintained under accepted germ-free conditions—i.e., by common cultural methods they were free from bacteria and fungi.[7-10] Excreta, food, water, and tank debris were routinely cultured, but only when the tank was entered for other purposes and at the end of the experiment. From the time of transfer at age 21 days until the experiment was terminated, the rats were kept on the following diet:

Casein (G. B. I.)	20 %	Corn starch	71 %
Salt mixture (Wesson)	4.0 %	Vitamin D_3 (oil soluble)	0.5 %
Corn oil	4.5 %	Thiamine hydrochloride	1 mg. %

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Pyridoxine hydrochloride	1 mg. %	B_{12}	10 γ %
Riboflavin	2 mg. %	Para-aminobenzoic acid	3 mg. %
Calcium pantothenate	2 mg. %	Folic acid	1.25 mg. %
Niacinamide	10 mg. %	Ascorbic acid	10 mg. %
Biotin	5 γ %	Menadione	0.2 mg. %
Choline chloride	200 mg. %	α-tocopherol acetate	5 mg. %

After the diet ingredients were mixed, 250 cc. of water per kg. of diet were added. The diet was then made into rolls and autoclaved at 250° to 255° F. for 30 minutes in the steam lock of a Reyniers tank, from whence it was passed into the plastic tank via a chemical lock (aerosol fog of 2 per cent peracetic acid with detergent). Food and water were supplied *ad libitum*.

In addition, 10 conventional non-germ-free Lobund rats of the same age and sex were maintained on the same diet, but outside the tank, being housed separately in wire-bottom cages. Both the germ-free and control groups were kept on the experimental regimen until 80 per cent of the animals in each group had died; the remaining rats were then sacrificed.

Tissues were fixed as soon as possible after death in 10 per cent buffered neutral formalin. For histologic examination the tissues were embedded in paraffin and stained with azure A-eosin B. Selected sections were also stained by the periodic acid-Schiff (PAS), method, Perls's method for iron, the Dunn and Thompson technique and the Wilder reticulum stain.[11] Fat stains were done with oil red O on frozen sections.[12]

Results

Excellent descriptions of both the clinical course and the pathologic features of vitamin A deficiency in conventional, non-germ-free rats are readily available not only in individual articles,[2,5,13–16] but also in books.[17–19] Observations, therefore, unless specifically indicated, will be confined to *gnotobiotes*.* Germ-free conditions were maintained throughout the experiment and all cultures were negative.

Clinical Observations

The germ-free animals continued to grow and to appear outwardly healthy until the 47th day on the diet, when one of the rats began to exhibit a slight squint and minimal periorbital crusting. By the end of another week, the squint and crust had become slightly more pronounced and the same change had begun to appear in several of the other animals. There were no grossly detectable corneal lesions. However, by this time all of the animals had begun to show slight weight loss, a rough, dull coat, and humped posture. Epilation, which was patchy, partial, and reminiscent of a "moth-eaten" type of alopecia, appeared and increased in severity. In the final stages of the disease, the rats developed a watery diarrhea which at times became profuse. Ac-

* A term coined by Reyniers to include the germ-free animals—the word being derived from the Greek stem *gnos*, known; and *bios*, life.[20]

tivity decreased and a fine tremor could frequently be seen while the animal was at rest. Although there was possibly slight ataxia and incoordination of movement, actual paralysis did not develop.

In spite of the emaciation and diarrhea, food and water consumption remained surprisingly constant, after having shown a decrease at the onset of symptoms. Toward the end of the experiment, spontaneous activity was for the most part confined to feeding. The rats had no apparent difficulty, however, in finding food, in mastication, or in deglutition. Anosmia did not develop; nor did priapism in the males.

The first death in the germ-free group occurred on the 82nd day of the experiment; and in the control group, on the 38th. Nevertheless, 4 control animals lived for 108 days, while 3 germ-free animals survived for 109 days. Average survival time for the 8 animals allowed to die from the disease was 82 days in the controls and 95 days in the germ-free group.

Gross Pathologic Observations

The germ-free group exhibited relatively few significant lesions. There was a decrease in but not complete absence of body fat. Lymphoid tissue was hypoplastic. Pituitary glands were quite small, about one half normal size. The livers appeared smaller than normal and varied in color from yellow-brown to rust. There was a slight increase in friability but no other abnormalities. Spleens also were small and somewhat browner than usual. Three rats exhibited renal calcification and hydronephrosis, but there was no accompanying ureteral or bladder dilatation. Another 2 animals had cystic dilation of the bladder which contained cloudy urine and flaky material. The preputial glands, though not enlarged, were multicystic. The stomach contained undigested food and tended to be dilated, as did the remainder of the intestinal tract. There was enormous dilatation of the cecum.

Microscopic Features

Skin. There was cystic atrophy of the sebaceous glands and associated hair follicles in those areas where the former are more concentrated—viz., the skin of the buccal pouch, the eyelid and the peri-anal region (Figs. 1 and 10). Skin changes in other areas were negligible. In no case was there hyperkeratinization of the skin, the hair follicles or sebaceous ducts.

Breast. Although sections of the mammary glands were not taken regularly, a definite proliferative reaction accompanied by cellular vacuolation suggestive of secretory activity was noted in the 4 females in which breasts were examined (Fig. 2).

Preputial Glands. There was marked cystic atrophy and stasis of secretion without apparent obstruction or hyperkeratinization of the excretory duct (Fig. 3). The acinar cells showed a partial to complete loss of perinuclear granules [21] so that many of the acinar cells resembled ordinary sebaceous cells (Fig. 4). The granules which did remain were weakly PAS-positive.

Salivary, Lacrimal and Orbital Glands. Squamous metaplasia was focal and primarily confined to the ducts, which occasionally were slightly dilated and contained keratinized debris along with a few leukocytes. Otherwise, the almost total lack of inflammation was striking, and accentuated even more the degenerative and necrotic alterations in the parenchymal cells. The submaxillary and parotid glands were the most severely affected and frequently were microscopically indistinguishable one from the other. Involvement of the major salivary glands tended to be uniform (Fig. 5), whereas the appearance of the glossal and tracheal mucosal glands (Fig. 6) varied from area to area. The lacrimal glands were less affected but did occasionally show focal areas of hyaline droplet degeneration (Fig. 7) and sometimes necrosis (Fig. 8). The harderian glands, except for a rare metaplastic duct, exhibited only absence of the usual pigmented secretion.

Eyes. The corneas without exception exhibited a thin superficial layer of keratinized cells (Fig. 9), but there was no evidence of inflammation or of vascularization. The conjunctivas showed foci of cornification (Fig. 10) although interspersed mucous cells were abundant. Among the internal ocular structures, degeneration of the retinal bacillary layer and underlying "pigmented" epithelium was noted (Fig. 11). Several of the retinas were detached in the sections, but this may have been artifactual.

Pituitary Glands. No characteristic cellular changes were noted, and castration cells [22,23] were absent.

Thyroid and Parathyroid Glands. There was questionable slight atrophy of the thyroid acini. This was associated with decreased colloid and occasional sloughing of the lining cells. The parathyroid glands were unremarkable.

Heart. In half of the germ-free animals, the ventricular myocardium exhibited focal areas of necrosis (Fig. 12) predominantly associated with an infiltration of mononuclear cells and, rarely, calcification. Four of the animals had died, but the lesion was also present in one of the rats killed at the termination of the experiment.

Respiratory Tract. There was patchy squamous metaplasia and keratinization of the larynx and trachea (Fig. 13); the bronchi and bronchioles were uninvolved. The lungs frequently showed vascular con-

gestion and, occasionally, perivascular edema. In only one animal was there alveolar edema, although two rats exhibited focal pulmonary hemorrhage.

Gastrointestinal Tract. An apparent increase in intraluminal mucus was noted throughout but was unaccompanied by discernible structural alteration in the mucosa, except for an occasional retention cyst with a few leukocytes at the base of a crypt. No gastric ulcers were seen.[24]

Liver. The most remarkable changes occurred in the liver. These varied from the presence of cytoplasmic hyaline droplets in occasional hepatic cells of the two sacrificed animals (Fig. 14) to actual liver necrosis in animals dying of the disorder (Fig. 15). The necrosis was coagulative in type and tended to involve individual cells, converting them into eosinophilic hyaline masses. Nuclear fragments were occasionally present, although frequently no nuclear remnants remained. Even with widespread involvement of the liver, the focal cellular nature of the injury was maintained, in that apparently viable cells were interspersed throughout (Fig. 16). There was total lack of any cellular inflammatory response to the necrotic tissue, no significant stromal collapse, and a negligible increase in reticulin. A moderate amount of fatty metamorphosis was noted in several livers and tended to be periportal in distribution. The hepatic parenchymal cells were pleomorphic and had vesicular but hyperchromatic nuclei with prominent nucleoli. One half of the animals also exhibited a peculiar type of tubular "degeneration" or transformation, manifest by ductlike arrangements of hepatic parenchymal cells (Figs. 15 and 17). In addition, proliferation of the bile duct epithelium was marked in most instances. This process extended from the portal areas and penetrated the lobule, the small ductular structures serpiginously intertwining among the hepatic cells (Figs. 18 and 19).

The common bile duct exhibited superficial squamous metaplasia and slight keratinization, but with associated parakeratosis (Fig. 20). The duct system was apparently unobstructed, and no evidence of bile stasis was noted.

The portal areas contained considerable deposits of an extracellular granular material consisting of pigments which stained green to brown with azure A-eosin B, gave a positive Prussian blue reaction for iron, stained with the PAS method, and to some extent with oil red O. This apparent combination of hemosiderins[11] and lipofuscins[11] was also present in small amounts within the Kupffer cells throughout the liver. There was no associated increase in fibrous connective tissue.

Spleen and Lymph Nodes. Although the lymphoid tissue proper, with the exception of Peyer's patches, was hypoplastic, hemosiderosis of the

spleen and abdominal lymph nodes was marked, but was unaccompanied by fibrosis.

Pancreas. The pancreas showed only minimal squamous metaplasia of occasional interlobular ducts without keratinization and without evidence of obstruction. In a few animals the major pancreatic duct exhibited changes similar to those noted in the common bile duct. No lesions were manifest in the islets and acini.

Adrenals. The adrenal glands were atrophic, had a thin zona glomerulosa, and exhibited a reduced amount of lipid on fat stain. In two animals the glands showed vacuolar degeneration of the zona glomerulosa; in two other rats there was extensive but focal bilateral cortical hemorrhagic necrosis (Fig. 21).

Urinary Tract. The kidneys of all of the animals exhibited mild degenerative changes such as cloudy swelling and fatty degeneration. In 4 rats, however, focal tubular necrosis (Fig. 22) was extensive, bilateral, and associated in 2 instances with pigmented casts in the convoluted and collecting tubules (Fig. 23). These casts gave a positive reaction with the Dunn and Thompson method for hemoglobin. In the more severely affected kidneys, occasional hyaline thrombi were seen in several glomeruli.

In addition to the above, 3 rats exhibited rather extensive tubular calcification and hydronephrosis (Fig. 24), the calcium being deposited in a PAS-positive matrix. In one rat the calcinosis and hydronephrosis were unilateral; in two, bilateral.

In no instance did the renal pelves show squamous metaplasia; nor did the ureters in the 3 animals examined. The bladder and particularly the urethra, however, did, in all cases, show considerable squamous metaplasia and keratinization.

Sex Organs. In males the testes were the site of marked tubular degeneration of both the Sertoli cells and the germinal epithelium. No spermatozoa were present. The tubular epithelium and basement membrane were separated from the interstitial tissue by protein-rich fluid (Fig. 25), and in one case by recent hemorrhage. The prostate glands exhibited atrophy, degeneration and focal squamous metaplasia. The epithelium of the seminal vesicles showed degranulation [25] and atrophy, along with some necrosis, but no metaplastic epithelial changes were noted. Mild cystic degeneration and focal metaplasia were found in the epididymis, and in one rat there was squamous metaplasia of the ductus deferens as well.

In females the vaginas were without exception heavily keratinized, but no leukocytic infiltration was present (Fig. 26). In the uterus the most extensive squamous metaplasia occurred in the glands near the

cervix but was also present focally within the endometrial glands of the cornua. Although "keratin pearls" were the rule, if plugging of a gland neck occurred, an exudation of leukocytes into the obstructed gland was not uncommon (Fig. 27). The superficial endometrium showed atrophy and vacuolar degeneration, but was not metaplastic. There was a striking absence of eosinophils in the endometrial stroma. In no case was there squamous metaplasia of the fallopian tubes. The ovaries of all 7 females had multiple large and prominent corpora lutea; the presence of spindle cells interspersed among the lutein cells suggested that none of these bodies were of recent origin. In addition there was an apparent absence of developing follicles as well as actual atresia of those already present. In all instances characteristic cart-wheel "deficiency" nuclei[26] were present in the surrounding thecal cells (Fig. 28).

Nervous System. Sections of the brain were essentially normal, except for some acute neuronal degeneration and cell shrinkage. Peripheral nerve ganglia and branches of peripheral nerves present in other sections were also not remarkable with conventional histologic techniques. An extensive histologic examination of the nervous system, however, was not carried out. (The teeth were given to the National Institute of Dental Research.)

Control Animals

In general, the control rats exhibited the usual accepted stigmas of A deficiency—i.e., widespread keratinizing metaplasia. Infection and inflammation, particularly of the tongue, salivary glands, respiratory and urinary tracts, were invariably present. Except for slight hemosiderosis, the livers appeared histologically normal, as did the kidneys. The adrenal glands were somewhat atrophic, depleted of lipid, and in two instances contained occasional colloid droplets in the cells of the zona glomerulosa; there was no hemorrhage or necrosis. The ovaries in the conventional animals tended to be more atrophic than in the germ-free, but there was less arrested follicular development, although luteal remains appeared more involuted. Deficiency cells, however, were present in all cases. Changes in the preputial glands and skin resembled those in the germ-free animals; sections of the mammary glands were not obtained.

Discussion

It has long been accepted that tissues such as the liver, which ordinarily contain a high concentration of vitamin A,[27,28] exhibit the least morphologic alteration in A deficiency.[18] Of particular interest, there-

fore, are the striking hepatic lesions in the germ-free rats. In several instances the necrosis alone was of sufficient magnitude to have been at least contributory to, if not the cause of, death. The bizarre focal necrosis and hyalinization of liver cells bear no resemblance to the diffuse cell death of "dietary hepatic necrosis,"[29] and, in fact, are not duplicated in any known deficiency state. On the other hand, the cellular pleomorphism, the duct formation from hepatic cells, and the proliferation of bile duct epithelium are indicative of injury or a metabolic defect which has been operative over a prolonged period of time. Similar changes are seen occasionally in chronic choline deficiency[30] and as an effect of certain carcinogens.[31] Why such a lesion should occur in the A-deficient germ-free rat and be completely absent in control animals on exactly the same diet remains an enigma. It may be that an A deficiency in the absence of normal bacterial flora precipitates a deficiency of another substance, or it may be that the presence of bacteria is necessary for the degradation of certain toxic metabolites produced by the deficiency, and that in the germ-free state these are re-absorbed. The possibilities are infinite and any attempted explanation would be no more than speculative, for even a viral etiology could not be excluded.

Such sporadically occurring lesions as the hemoglobinuric-like nephrosis and renal tubular degeneration may or may not be due to the A deficiency *per se;* more likely they are related to liver necrosis and shock. Although focal hemorrhages can certainly occur with shock and anoxia, hemorrhagic necrosis of the adrenal gland is not usually seen. Adrenal hemorrhage and necrosis do occur in pantothenic acid deficiency,[32] but calcium pantothenate was given in sufficient amount to protect the ordinary germ-free rat from this condition. Menadione (vitamin K) was also supplied in ample quantity. Ascorbic acid, which is present in the adrenal gland[33] and which could conceivably be involved in a hemorrhagic diathesis, was added to the diet but may not have survived autoclaving. Although conventional and germ-free rats on normal diets have not as yet demonstrated a need for an exogenous source of this vitamin, it has been reported that in the conventional A-deficient rat there is a co-existent depletion of tissue ascorbic acid.[34] Other investigators[35] have claimed that the C depletion is related to the decreased food intake of the A-deficient animal. If the latter statement is true, then the A-deficient germ-free rat should not be affected, as food intake was apparently ample. Utilization, of course, is another matter, but this phase has not been investigated as yet. In this same regard, the close topographic association between vitamins A and C should also be remembered—that is, those sites having a high tissue concentration of vitamin A are frequently also high in C; e.g., the adrenal gland and the corpus luteum.[33,36]

In the germ-free rat as in the conventional animal, a deficiency of vitamin A results in widespread squamous metaplasia and keratinization. In the absence of secondary infection, however, such changes lose much of their pathogenic significance. "Xerophthalmia," long regarded as the pathognomonic sign of avitaminosis A, was represented only by a thin superficial layer of keratin. Interestingly enough, keratinization of the cornea and conjunctiva, as well as dilatation of the meibomian glands, has also been reported in sodium deficiency.[37] Even the heavily keratinized vagina, which is generally accepted as one of the earliest manifestations of the disease,[38] presented no distinctive characteristics and was completely devoid of the leukocytic infiltration which has been said to distinguish the A-deficient from the estrogen-stimulated vagina.[39,40]

There were no tongue abscesses to interfere with swallowing.[2,41] There was no purulent rhinitis or nasopharyngitis to produce anosmia[5]; no ulcerative urethritis, common in conventional animals, to account for priapism[5]; and inflammatory lung lesions were nonexistent. The occurrence of "bronchiectasis" secondary to the desquamation of keratinized epithelium[5] was not observed. In fact, even in those organs such as the salivary glands, which were severely involved, degeneration and necrosis of the parenchyma—not simply atrophy—far overshadowed ductular keratinization. The interstitial infiltration by lymphoid and plasma cells observed by Wolbach and Howe[5] in the reported absence of infection was not seen. Except for a few leukocytes in an occasional apparently plugged duct, inflammatory reactions of any kind were rare.

In those tissues and organs not ordinarily susceptible to "keratinizing metaplasia," infection in conventional animals is extremely unusual. Other alterations do occur, however, which reflect the generalized nature of the underlying disturbance, but which, because of the more striking epithelial changes, have in the past been minimized. Less specific, to be sure, but still significant, are the focal lesions in the heart, which Wolbach and Howe[5] observed in 16 of 19 rats. In spite of the occurrence of somewhat similar lesions in other conditions—e.g., potassium deficiency,[42] thiamine deficiency[43] and biotin deficiency[44]—the foci of myocardial necrosis are nonetheless a part of A deficiency. In the germ-free animals, lesions were present in half of those dying and in one of the killed rats. The cardiac lesion, therefore, is probably neither necessarily lethal in effect nor agonal in origin. In fact, the presence of calcium in some of the foci suggests but is not proof for some degree of chronicity.

In regard to retinal degeneration, our findings are in general agreement with the observations reported by Johnson.[45] They are not as severe, since only the bacillary layer and the "pigmented" epithelium

were involved, and no "rosettes" were formed. Johnson found that lesions involving the rod and cone layer were reversible, but that if there was involvement of the greater part of the outer nuclear layer or more, the damage was irreparable.[46] By fluorescence microscopy, vitamin A is localized in the bacillary and pigmented epithelial layers of the retina.[47]

Cystic atrophy of sebaceous glands and hair follicles was striking; whether these adnexal changes bear any relationship to the acquired alopecia, except perhaps in certain localized areas, is doubtful. Deficient rats, regardless of the specific deficiency, often exhibit a pica-like trichophagy[48] in an apparent attempt at diet supplementation. In any event, the skin changes bear no histologic resemblance whatsoever to such human conditions as Darier's disease, keratosis pilaris or keratosis follicularis, for which a relationship to vitamin A deficiency has been postulated.[49] This association between vitamin A and human hyperkeratosis was first suggested by applying the experimental findings of Wolbach and Howe[5] to cutaneous lesions in man.[50] However, not only did Wolbach and Howe confine their experimental observations in rats to the skin of the eyelid and base of the ear, but they also reported "no striking changes."[5] Hyperkeratinization of the epidermis does not occur in A-deficient rats.[51] The idea has, nevertheless, persisted in regard to human A deficiency, although hyperkeratotic skin lesions have not been produced in experimental A deficiency in man,[52] nor have they been reported in necropsied cases.[53] It is true that the latter, for the most part, consist of infants and children with fibrocystic disease of the pancreas. In these, because of squamous metaplasia at various sites, A deficiency was assumed to be present; therefore, they may not be valid examples. In fact, the microscopic description of the first necropsied human case of vitamin A deficiency[54] would lead one to believe that the patient not only had cystic fibrosis of the pancreas, but salivary gland inclusion disease as well. Interestingly enough, it is this complicated case which has been cited as being comparable to the disease in rats.[5]

Since cystic atrophy occurs in the skin adnexal structures, it is not surprising that the preputial gland, which embryologically is derived from the skin[55] and which normally contains vitamin A,[56] should take part. The peculiar evanescence of the perinuclear granules, however, is an interesting phenomenon, since the granules themselves ordinarily share some of the histochemical reactions of keratin[55] and might be expected to increase in prominence. Why the few remaining granules become PAS positive when ordinarily they are not stained by this method is also unexplainable. Except for the normal glandular size and the acinar

cell degranulation, the cystic atrophy resembles that seen following prolonged testosterone stimulation.[55]

It has long been recognized that vitamin A is in some way intimately concerned with reproduction.[57] The exact mechanism is unknown. The large corpora lutea, the lack of follicular development, and the stimulation of breast tissue which were seen in the germ-free animals may not be significant but are interesting in that they fall into an endocrinologic pattern, i.e., all of the changes could be brought about by any substance capable of producing a functioning corpus luteum. The reported occurrence of spontaneous deciduomas in A deficiency [58] is interesting in this same regard. On the other hand, although the appearance of ovarian deficiency cells is generally accepted as being due to a decreased output of pituitary gonadotropin,[59] the A-deficient pituitary has been said to contain an increased number of basophils [60] and to exert above normal gonadotropic activity.[61] If this is true, and if uterine squamous metaplasia follows avitaminosis A only when the epithelial cells are under the influence of estrogen,[62] what was its source and why was there no accompanying infiltration of the endometrial stroma with leukocytes? [63] At the present time it is impossible to account for all of these endocrinologic aberrations, since some are mutually exclusive.

The failure of either the germ-free or control animals to develop paralysis cannot be explained. According to Wolbach and Bessey,[64] if weanling rats were started on an A-deficient diet, paralysis almost invariably appeared between 6 and 9 weeks of age, generally beginning about the time weight gain fell below normal. It was thought that this paralysis was due to a mechanical phenomenon caused by disproportionate growth between the central nervous system and the skeletal system although no discernible changes could be demonstrated in the bone itself. Though probably not significant, it should, nevertheless, be mentioned in passing that during the early days of investigation, several dietary factors, including B_{12}, were unknown and that brewers' yeast frequently served as the only source of vitamin supplementation.

In still another category are lesions which have been reported in A deficiency, but which also occur in the nondeficient germ-free state—e.g., hemosiderosis of the liver and spleen, and renal calcinosis. It is impossible to tell whether the final effect in the A-deficient germ-free rat has been additive or synergistic. To be sure, the hemosiderosis is much more marked than that seen in A deficiency alone or in the occasional, generally older, germ-free animal killed at random. Renal calcinosis with hydronephrosis, on the other hand, was no more severe than that seen in germ-free rats in general, but did occur at a much younger age. In fact, renal calcification has even been reported in hyper-

vitaminosis A.[65] Lymphoid hypoplasia and dilatation of the cecum, of course, are products solely of the germ-free environment, and bear no relationship to A deficiency at all.

One of the more surprising aspects of the experiment was in regard to survival time. In the germ-free group 3 animals lived for 109 days and in the control group 4 rats lived for 108 days. In spite of the severe liver, kidney and adrenal lesions in the former, and the constant presence of suppurative infection in the latter, survival time was not appreciably altered, and, in fact, compared favorably with the 61 to 109 day survival originally reported by Wolbach and Howe for a similar group of young rats.[5]

Summary

In addition to the usual stigmas of vitamin A deficiency, the germ-free rats exhibited severe hepatic, renal, and adrenal lesions, which were not present in conventional rats on an identical diet. In other organs, the lesions in the germ-free group resembled those in non-germ-free animals, but were modified by the absence of infection and inflammation, so that atrophy, degeneration, and necrosis became the predominant features of the disease, although focal keratinizing metaplasia did occur. Death supervened independently of the germ-free state, and survival time was not increased.

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[*Illustrations follow*]

Legends for Figures

All illustrations are of tissues obtained from vitamin A-deficient germ-free rats. The photomicrographs were prepared from sections stained with azure A-eosin B.

Fig. 1. Skin of the buccal pouch. There is marked cystic atrophy of sebaceous glands and hair follicles. Note the lack of hyperkeratinization. × 18.

Fig. 2. Mammary gland. Acinar proliferation and apparent secretion are evident. × 120.

Fig. 3. Preputial gland. There are cystic atrophy and stasis of secretion. × 18.

Fig. 4. Preputial gland. Degranulation of acinar cells produces a resemblance to the ordinary sebaceous gland. Perinuclear protein granules are still present in some of the cells in the upper portion of the photograph. × 210.

Fig. 5. Salivary glands: parotid, upper left; sublingual, upper right; submaxillary, lower right. Atrophy and degeneration are accompanied by focal squamous metaplasia of the ducts. Note the total absence of inflammation and edema. × 18.

Fig. 6. Trachea. Mucosal glands exhibit cystic atrophy; the superficial epithelium is also atrophic. × 105.

Fig. 7. Lacrimal gland. Hyaline droplet degeneration is evident in the upper left. There is ductular transformation of an acinus (right) and early squamous metaplasia of a duct (lower right). × 335.

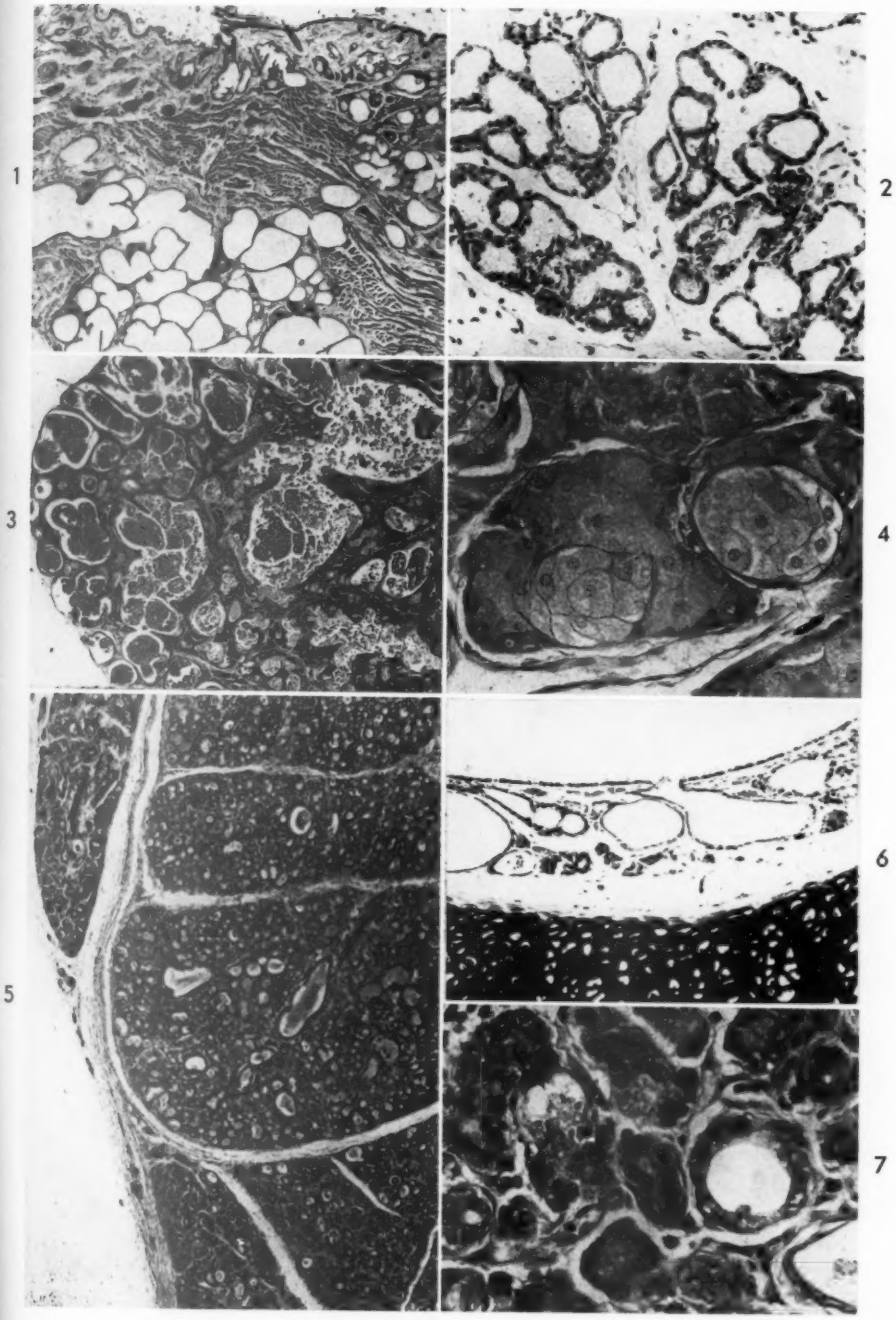


FIG. 8. Lacrimal gland. Clumping of chromatin and karyorrhexis are manifest. × 435.

FIG. 9. Cornea. Superficial keratinization is associated with a complete lack of inflammation and vascularization. × 125.

FIG. 10. Eyelid. Keratinization of the palpebral conjunctiva appears on the left. There are atrophy and cystic dilatation of the meibomian glands. × 18.

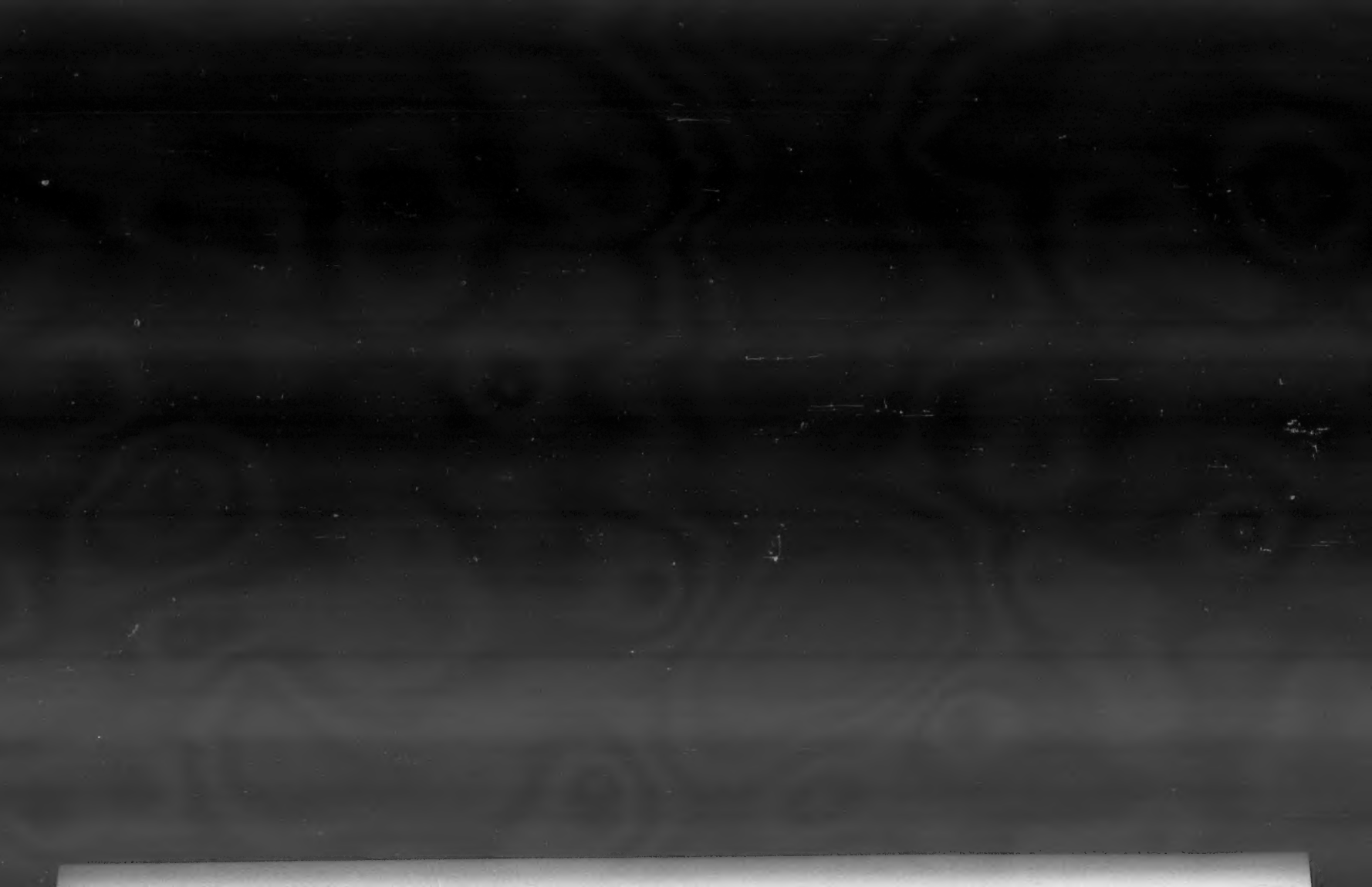
FIG. 11. Retina. Degeneration of bacillary layer and sloughing of "pigmented" epithelium are shown. × 125.

FIG. 12. The ventricular myocardium is the seat of focal necrosis. × 65.

FIG. 13. Trachea. Superficial squamous metaplasia and keratinization are unaccompanied by inflammation. The large dark-staining cells (left) are mast cells. × 250.

FIG. 14. Liver. Cytoplasmic hyaline droplets appear in parenchymal cells. × 775.

FIG. 15. Liver. Isolated cell necrosis is characterized by pyknotic stellate cells (off center) and karyorrhexis (lower right). Duct formation by hepatic parenchymal cells is also evident. × 250.



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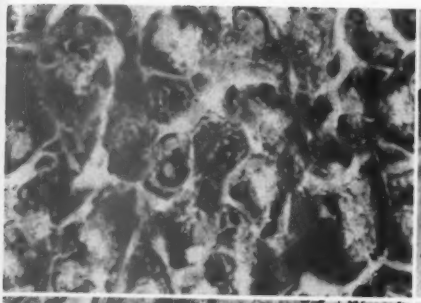

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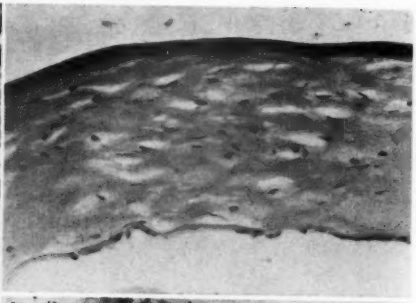

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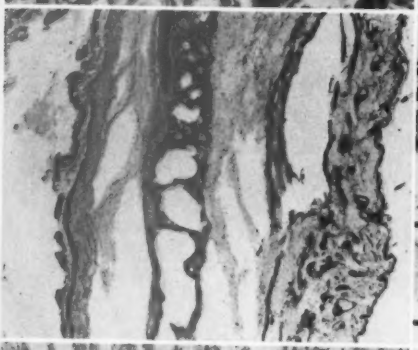

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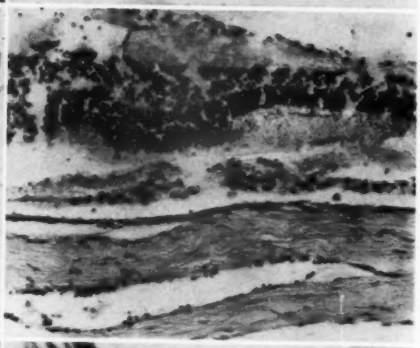

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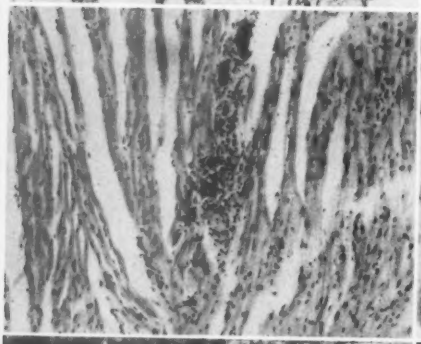

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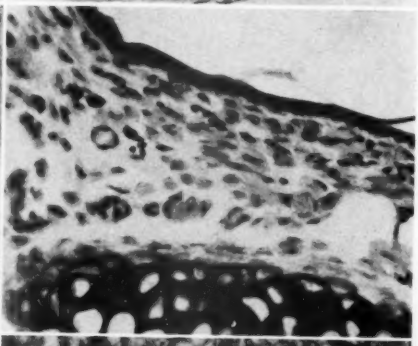

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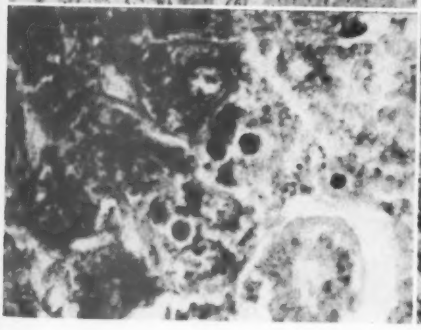

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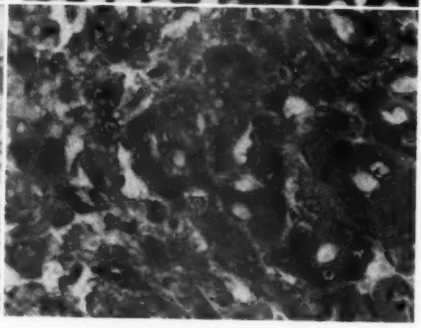

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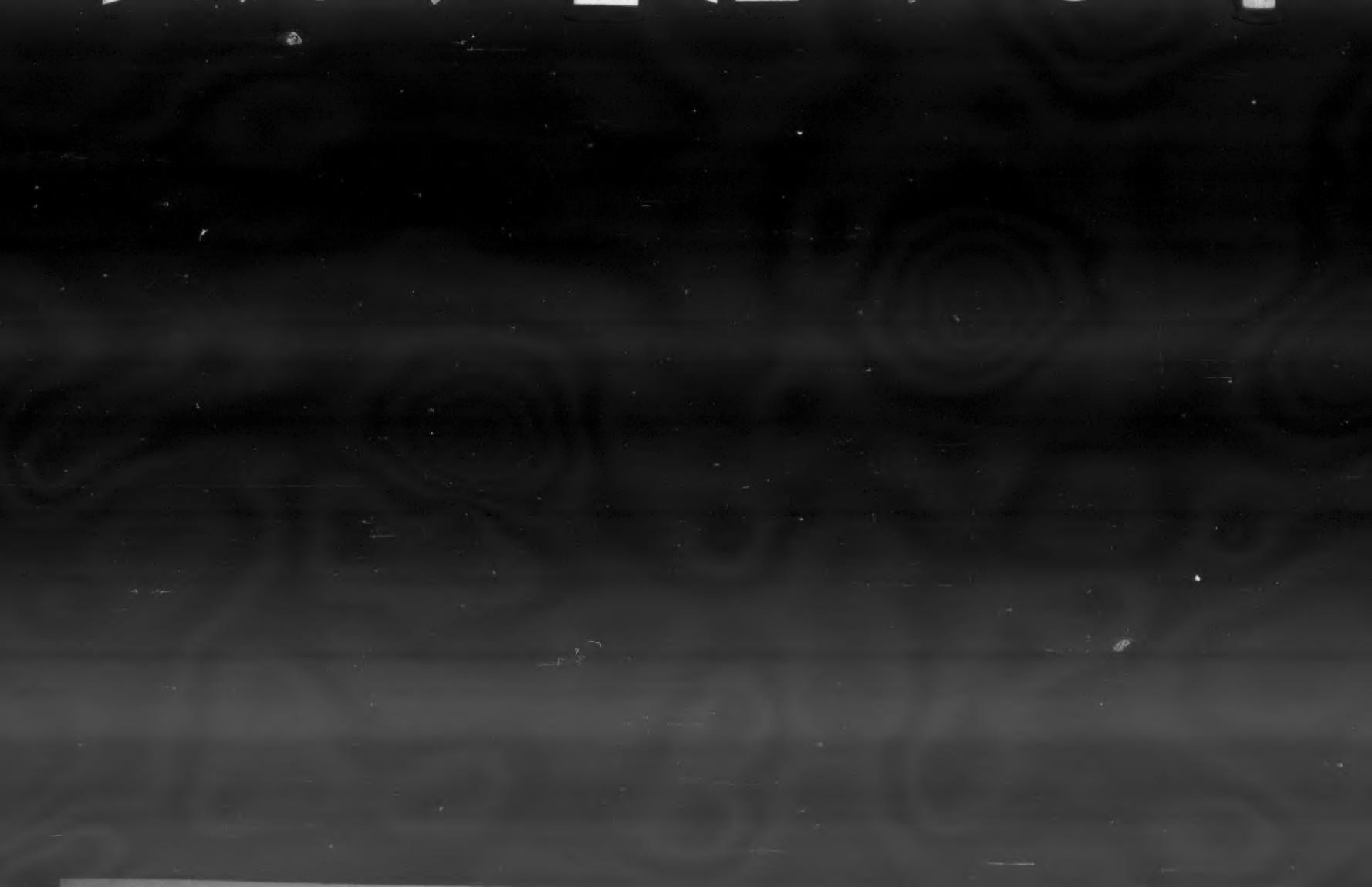
FIG. 16. Liver. There is extensive necrosis with apparently viable hepatic cells interspersed throughout. Occasional inclusion-like cytoplasmic hyaline droplets persist in necrotic cells (arrow). Compare with Figure 14. × 290.

FIG. 17. Liver. Ductlike structure appears to be formed by hepatic parenchymal cells. Note also the nuclear pleomorphism of adjacent cells. × 250.

FIG. 18. Liver, portal area. There is striking proliferation of bile duct epithelium. × 143.

FIG. 19. Liver. A higher magnification of same area shown in Figure 18. × 235.

FIG. 20. Common bile duct. Superficial squamous metaplasia and keratinization with parakeratosis are shown. × 130.



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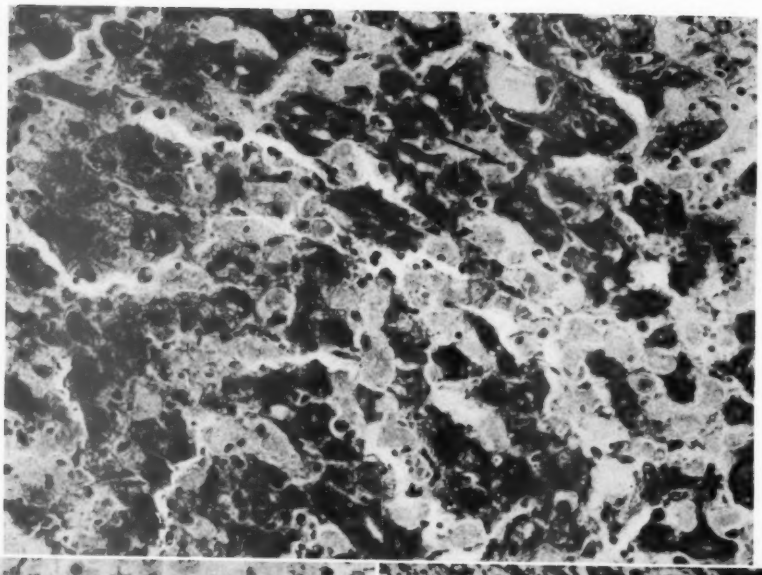

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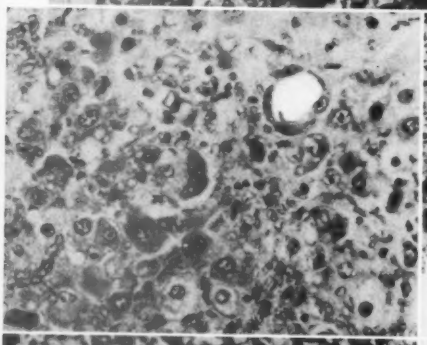

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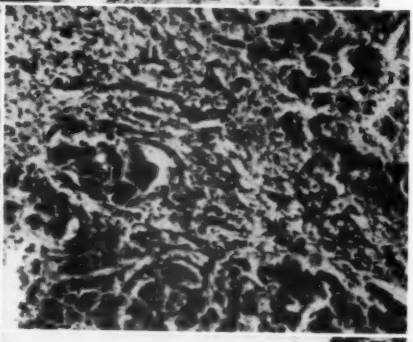

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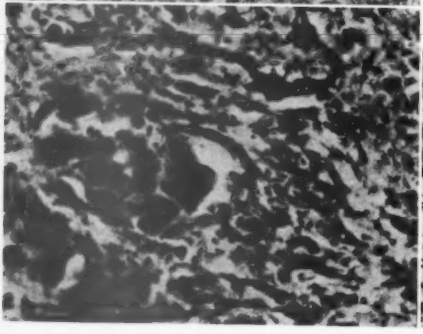

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FIG. 21. The adrenal cortex is the seat of hemorrhagic necrosis (left). × 65.

FIG. 22. The kidney shows focal cortical tubular degeneration and necrosis. × 125.

FIG. 23. Kidney. A collecting tubule contains a pigmented granular cast. × 250.

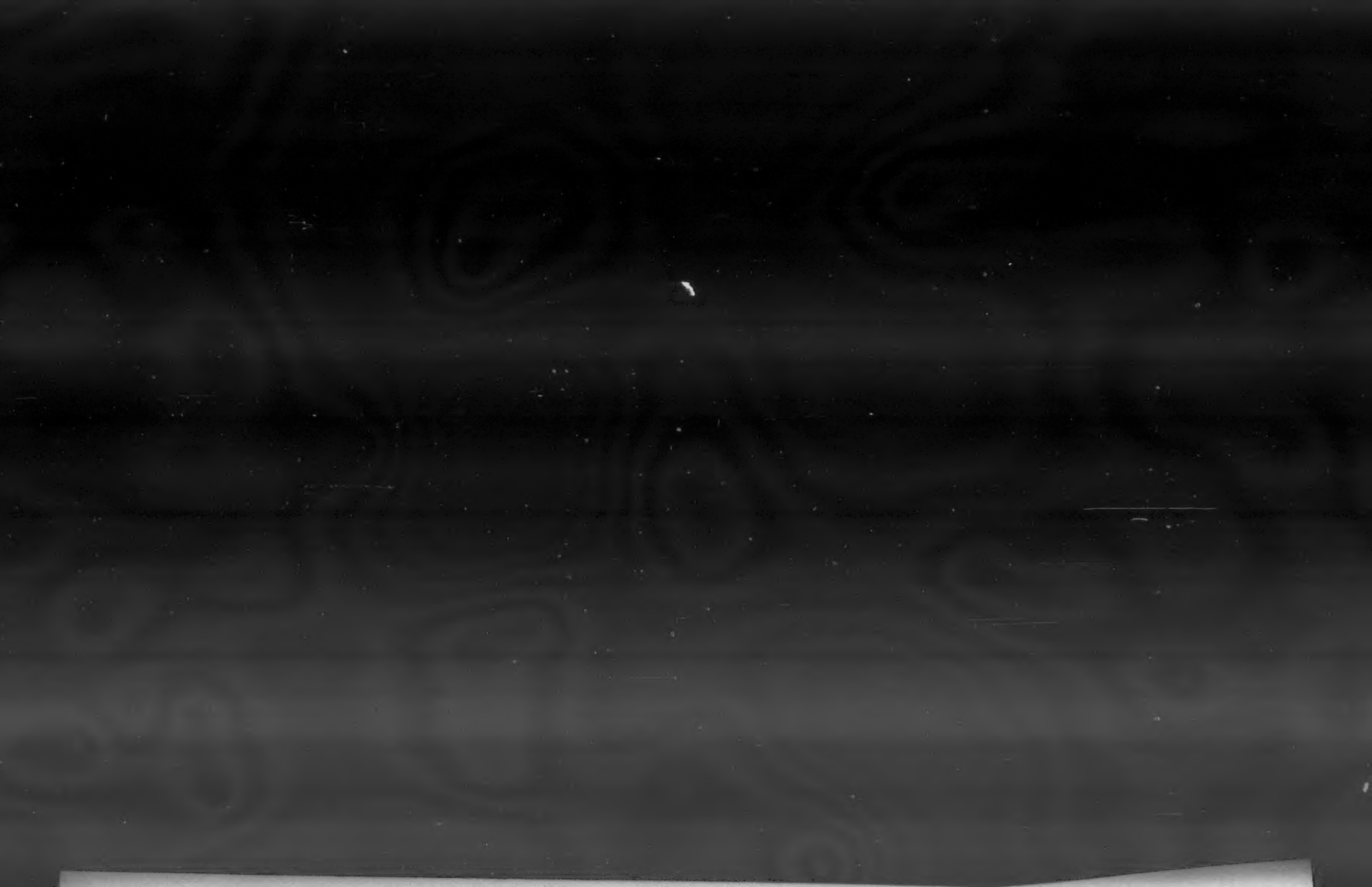
FIG. 24. The kidney exhibits calcinosis and hydronephrosis. There is a dilated calyx on the right. Note the absence of squamous metaplasia. × 18.

FIG. 25. A testis shows severe tubular degeneration and edema. The protein-rich fluid apparently has accumulated between the basement membrane and the interstitial tissue. × 50.

FIG. 26. The vagina is characterized by marked hyperkeratinization; there is no leukocytic infiltration. × 36.

FIG. 27. Uterus. A dilated keratinized endometrial gland exhibits a blocked duct and an intraluminal inflammatory exudate. Vacuolar degeneration has occurred in the superficial endometrium. × 110.

FIG. 28. Ovary. "Deficiency" cells are shown. × 775.

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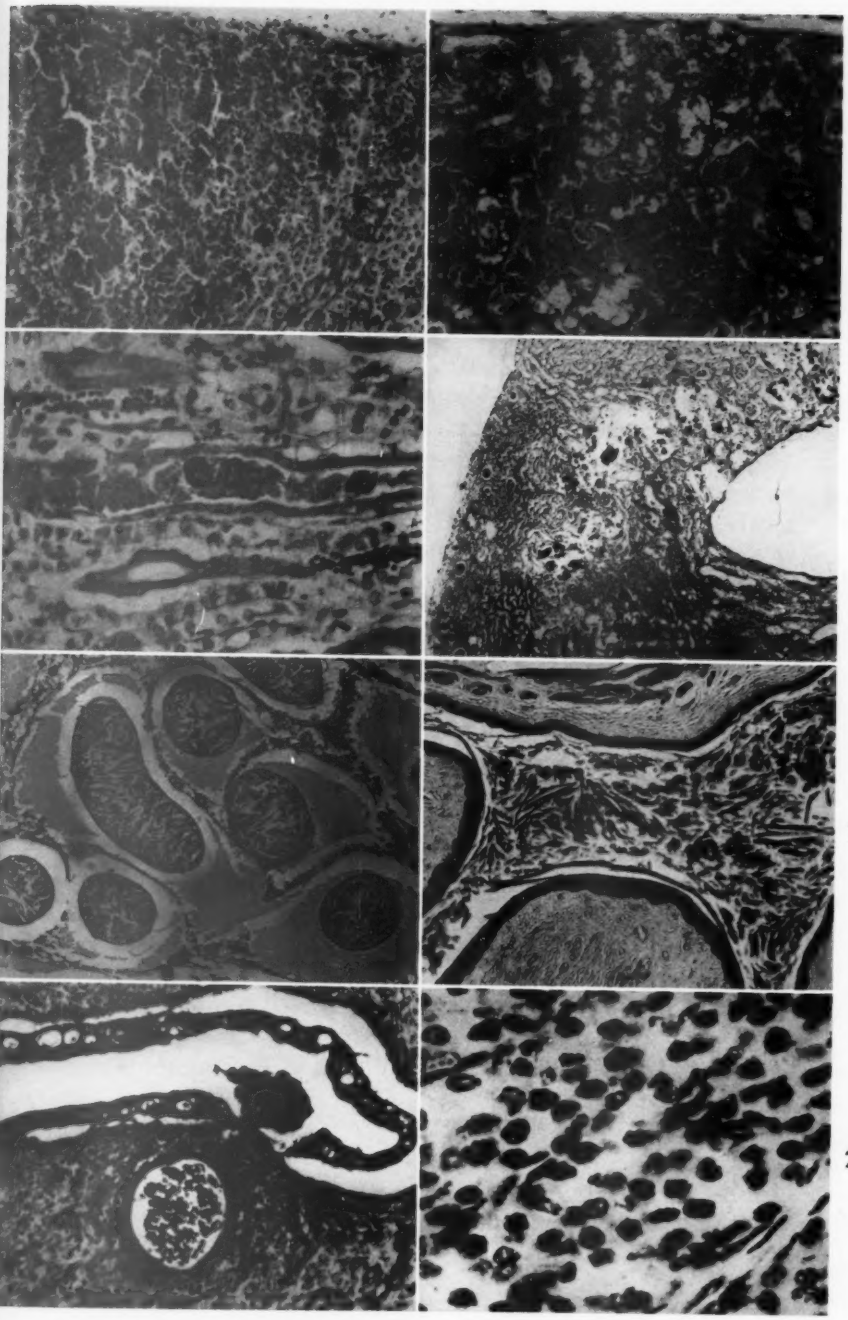
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RENAL NEOPLASMS IN THE IRRADIATED AND NONIRRADIATED SPRAGUE-DAWLEY RAT

V. J. Rosen, M.D.*; T. J. Castanera, M.S.;
D. J. Kimeldorf, Ph.D., and D. C. Jones, Ph.D.

From the Biological and Medical Sciences Division, United States Naval Radiological Defense Laboratory, San Francisco, Calif.

Although rats are known to exhibit a wide spectrum of neoplasms, primary tumors of the kidney have been thought to be rare in this species. Curtis, Bullock and Dunning[1] studied 2,245 pedigreed rats bred from 7 strains and reported 4 renal tumors, consisting of 2 embryonal carcinosarcomas, a carcinoma, and a sarcoma. In a survey of approximately 100,000 wild rats, McCoy[2] found 11 renal tumors, but detailed histologic descriptions were not given. Gilbert and Gillman[3] examined 1,342 Wistar rats and reported 3 renal neoplasms. Ratcliffe[4] reported 4 embryonal and 1 undifferentiated malignant renal tumor in a sample of 486 Wistar rats, while Crain[5] reported 1 papillary cystadenoma, 1 lipomatous hamartoma, and 1 embryonal tumor in the kidneys from 786 Wistar rats. Eker[6] reported several familial renal adenomas in a strain of Wistar rats, but exact figures as to incidence were not given.

Koletsky and Gustafson,[7] investigating the effects of whole-body irradiation on carcinogenesis, reported 8 renal cortical tumors in 123 irradiated (660 r) male Wistar rats with a mean life span of 417 days, as opposed to no renal neoplasms in 36 controls having a mean life span of 636 days.

During a study of the long-term effects of ionizing radiation on Sprague-Dawley rats in this laboratory, it was found that over one third of the irradiated population had primary renal tumors, while such neoplasms were not observed in controls. The tumors proved to be cortical adenomas and carcinomas, as well as transitional-cell neoplasms of the renal pelvis. It is the purpose of this report to describe the various renal neoplasms encountered in the irradiated Sprague-Dawley male rat and to discuss certain radiobiologic implications of these tumors.

Materials and Methods

The animals were male rats of the Sprague-Dawley strain from a stock bred for several generations at this laboratory. The animals were individually caged and

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* Lieutenant (MC), United States Naval Reserve.

maintained in air-conditioned rooms. A standard isolation gown and scrub technique were used during the handling of the animals, and no epidemic respiratory infections were observed throughout the investigation.

The rats were 101 days of age at the time of irradiation. Total body x-ray exposures were made with a 250 KVCP Westinghouse deep therapy unit, operated at 15 ma, using a 0.5 mm. Cu and 1 mm. Al filter (HVL, 1.5 mm. Cu). The dose rate was 27 r per minute (air) as measured by a Victoreen Chamber. A factor of 0.97 was used to convert the roentgen dose, measured in a paraffin phantom, to rad dose. High sublethal (430 rad) and approximately midlethal (680 rad) exposure doses were used.

The animals were followed for their entire life spans, and finally, at death, all rats were examined at necropsy, and tissues were removed for subsequent microscopic examination. All kidneys were sectioned for microscopic study. Tissues were fixed in formalin-acetic acid-alcohol solution and embedded in paraffin. Sections were cut at 4 μ and stained with hematoxylin and eosin.

Observations

The various types of tumors will be described separately. The incidence of the renal neoplasms is shown in Table I. The specific tumor distribution is shown in Table II. The combined incidence of 3 renal neoplasms was 34 per cent among the irradiated rats, whereas the non-

Table I

INCIDENCE OF RATS HAVING RENAL NEOPLASMS

Rad dose	No. of rats	No. of rats with renal tumors	%
0 rad (controls)	41	0	0
430 rad (x-ray)	42	17	40
680 rad (x-ray)	58	17	29

Table II

SPECIFIC NEOPLASM ENCOUNTERED

	430 rad	680 rad
Cortical adenoma	8	11
Cortical carcinoma	6	2
Transitional cell carcinoma	4	6

irradiated control population derived from the same litters failed to exhibit any primary tumors in the kidney. No significant dependent relationship between radiation dose and the incidence of renal neoplasms was apparent from these data. One rat had 3 morphologically different tumors within the same kidney: an adenoma, a cortical carcinoma and a transitional cell tumor. One animal had a cortical carcinoma in the right kidney and an adenoma in the left one. Two additional rats each had 2 discrete adenomas in 1 kidney. Thus the 34 tumor-bearing rats had a total of 39 separate renal neoplasms.

The median life span of the control animals in this study was 697 days, while that of the animals receiving 430 rad was 613 days, and that of the animals receiving 680 rad was 529 days. The age ranges of the animals having renal tumors at death was 408 to 820 days. Renal adenomas were found in animals dying from 424 to 731 days, with a median age of 586 days, while renal carcinomas were found in rats dying from 569 to 820 days, with a median age of 640 days. The transitional cell tumors were found in animals dying from 408 to 715 days, with a median age of 600 days. These age ranges for the appearance of renal neoplasms are especially interesting since 17 irradiated animals died between 222 and 407 days of age and exhibited no gross or histologic evidence of renal tumors of any kind. In addition it should be noted that for rats surviving more than 400 days, the tumor incidences are 46 and 37 per cent for 430 and 680 rads, respectively.

Although all of the animals examined had chronic renal disease, the severity of the disorder was quite variable, even in older animals, ranging from minimal microscopic foci to extensive gross alterations. Extensive renal lesions were found no more frequently in the animals with tumors than in those without them. The various patterns of the renal alterations encountered were indicative of glomerulitis, pyelonephritis, and vascular sclerosis, in various combinations. These will be described in detail in a forthcoming report.

Cortical Adenoma

The 21 individual adenomas presented as discrete, oval to spherical, pale tan to white nodules usually located within the subcapsular cortex. They measured from 0.2 to 0.4 cm. in greatest dimension.

Microscopically, encapsulation was prominent, and cellular pleomorphism was not observed. Many different patterns were present, and most adenomas showed blending of multiple cell patterns (Figs. 1 and 2). Many tumors were cystic, lined by prominent papillary folds covered with cuboidal to columnar, moderately basophilic cells while other papillary projections were covered with eosinophilic cells resembling "oncocytes." Other fields were composed of solid sheets of eosin-staining cells (Fig. 3). In other areas sheets of clear cells appeared to blend with solid cords of basophilic cells. These clear cells bore a striking resemblance to those observed in the clear cell renal adenocarcinoma in human beings. Abortive tubule formation was occasionally observed. Small areas of necrosis were present, and flecks of calcification were scattered through many of the neoplasms.

One kidney with a large cortical carcinoma contained, in a tubule dis-

tant from the neoplasm, a curious proliferation of clear cells (Fig. 4). Kidneys from other irradiated animals exhibited scattered dilated tubules containing early papillary proliferations of eosinophilic lining cells (Fig. 5).

Renal Cortical Carcinoma

Eight tumors were classified as cortical carcinoma. These measured from 0.4 to 1.2 cm. in greatest dimension and were grayish tan to pale yellow. The diagnosis of carcinoma was made when the neoplasm exhibited abundant nuclear atypicality and evidence of invasion into the surrounding renal parenchyma. It was, of course, difficult to separate clearly the cortical adenomas from the carcinomas. Visceral and lymph node metastases were not observed in animals with cortical carcinomas. In the strictest sense, the assignment of the label carcinoma is somewhat arbitrary. Disruption of the overlying renal capsule and invasion of the perirenal soft tissues were seen in two cases; in one of these there was massive retroperitoneal hemorrhage.

Histologically, the carcinomas exhibited the various patterns described in the cortical adenomas, but showed a larger clear cell component. A few of the tumors were composed, in part, of large areas of papillary growths of clear cells within cystic spaces. Necrotic foci were common (Figs. 7 and 8). One cortical carcinoma was composed of spindle-shaped cells interspersed with large clear cells and contained, also, several pseudoglomerular structures and clefts (Fig. 7).

Transitional Cell Carcinoma

The 10 transitional cell tumors appeared as gray, firm, coarsely granular, sessile lesions related to the lining mucosa of the renal pelvis. Papillary outgrowths were usual but gross infiltration deep into the renal substance was prominent in 7 of the 10 tumors. Three lesions were recognized macroscopically by their surface granularity. The tumors varied from 0.4 to 2.1 cm. in greatest diameter. Three renal pelves were entirely filled with neoplasm, and there was an accompanying suppurative pyelitis. Calcific calculous debris was identified within the pelves of 6 of the kidneys with transitional cell tumors.

Microscopically, the lesions were composed of fairly well differentiated, multi-layered, transitional epithelium having, generally, a more basophilic staining reaction than normal pelvic mucosa. The larger, bulky tumors showed cellular necrosis in the central portions.

Early infiltration was recognized in even the smallest neoplasm (Figs. 10 and 11). Frank blood vessel invasion was not identified, although lymphatic invasion was seen in two of the larger masses. One of these

was very well differentiated. No distant metastases were found, but retroperitoneal nodes contained tumor cells in one instance.

Discussion

In light of the data presented, it appears that radiation is a potent renal carcinogenic agent in the male Sprague-Dawley rat. Renal neoplasm was not encountered in senile, nonirradiated control rats, and a very low incidence of spontaneous renal neoplasm in the rats has been reported in the literature. Thus, the striking incidence in both irradiated groups in the present study is remarkable. In considering the late effects of ionizing radiation, it is sometimes convenient to regard irradiation as an aging treatment; thus, an irradiated animal is held to be physiologically or pathologically comparable to a chronologically older nonirradiated control. This viewpoint is not useful when the parameter is the incidence of primary renal tumor in the rat since these tumors are, at the most, extremely rare in the nonirradiated animal, irrespective of age. While one may argue the statistical inevitability of an appreciable incidence of renal neoplasms in control rats provided they survive long enough, such a hypothesis appears to have little validity in view of the life spans actually encountered in experimental work with the rat.

The renal cortical adenomas and carcinomas are quite similar, so that it is not possible to effect a sharp separation. The animals with "carcinoma" had a median age at death approximately 50 days older than those with adenoma. The carcinoma probably represents a later stage of the adenoma, which, in turn, appears to arise from renal tubular epithelium.

Some renal cortical tumors were observed in areas with no evidence of tubular obstruction or inflammation although others were found within regions of scarring and chronic alteration. It appears of special interest that some old nonirradiated rats had severe, destructive renal lesions, yet their kidneys showed no evidence of neoplasm.

Transitional cell tumors, however, were not observed in kidneys with mild renal disease. All animals with these neoplasms had chronic inflammatory lesions. It is possible that the neoplasms provoked the chronic renal lesions by obstruction and inflammation. Previous reports of transitional carcinoma of the kidney in the rat following irradiation could not be found.

Summary

A high frequency of renal neoplasm was observed in irradiated Sprague-Dawley rats. Seventeen (40 per cent) of 42 rats receiving 430 rad (x-ray) and 17 (29 per cent) of 58 rats receiving 680 rad (x-ray)

had renal tumors. No renal tumors were observed in 41 control animals.

The lesions encountered were cortical adenoma (19), cortical carcinoma (8), and transitional cell carcinoma (10), a total of 39 separate neoplasms in 34 rats.

It appears that radiation exerts a carcinogenic effect on the rat kidney, an organ rarely the site of neoplasm in the nonirradiated subject.

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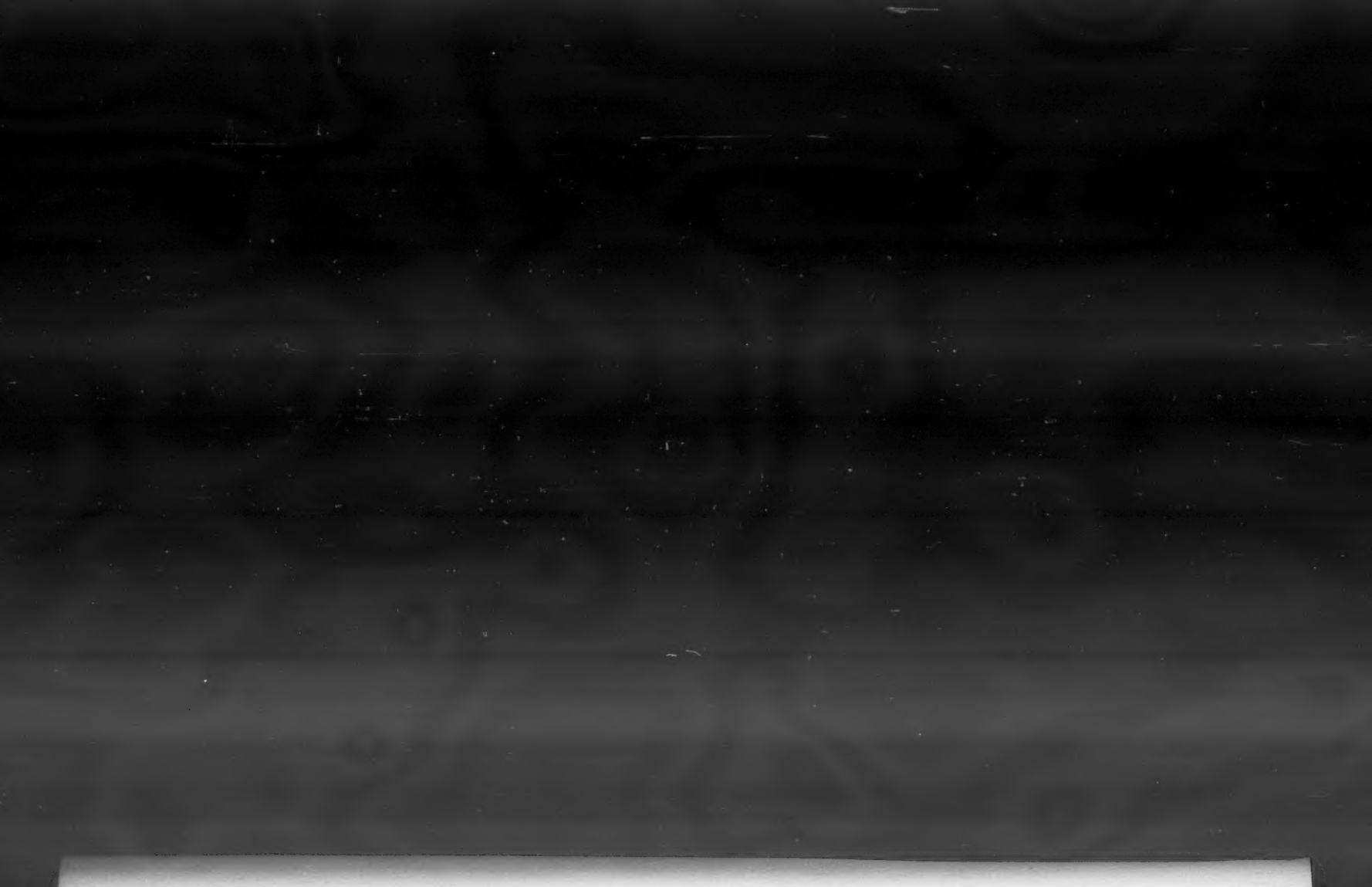
Legends for Figures

Photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Small cortical adenoma. The tumor is composed predominantly of papillary processes covered by small basophilic cells. A small, solid eosinophilic focus is seen in the lower portion of the tumor. × 120.

Fig. 2. Large cortical adenoma. The tumor is composed of solid eosinophilic cells with occasional groups of clear cells. Several abortive attempts at tubule formation are seen. A small papillary cystic structure is manifest. × 70.

Fig. 3. Solid eosinophilic adenoma. A few vacuolated cells are evident, and scattered nuclei are also vacuolated. × 160.



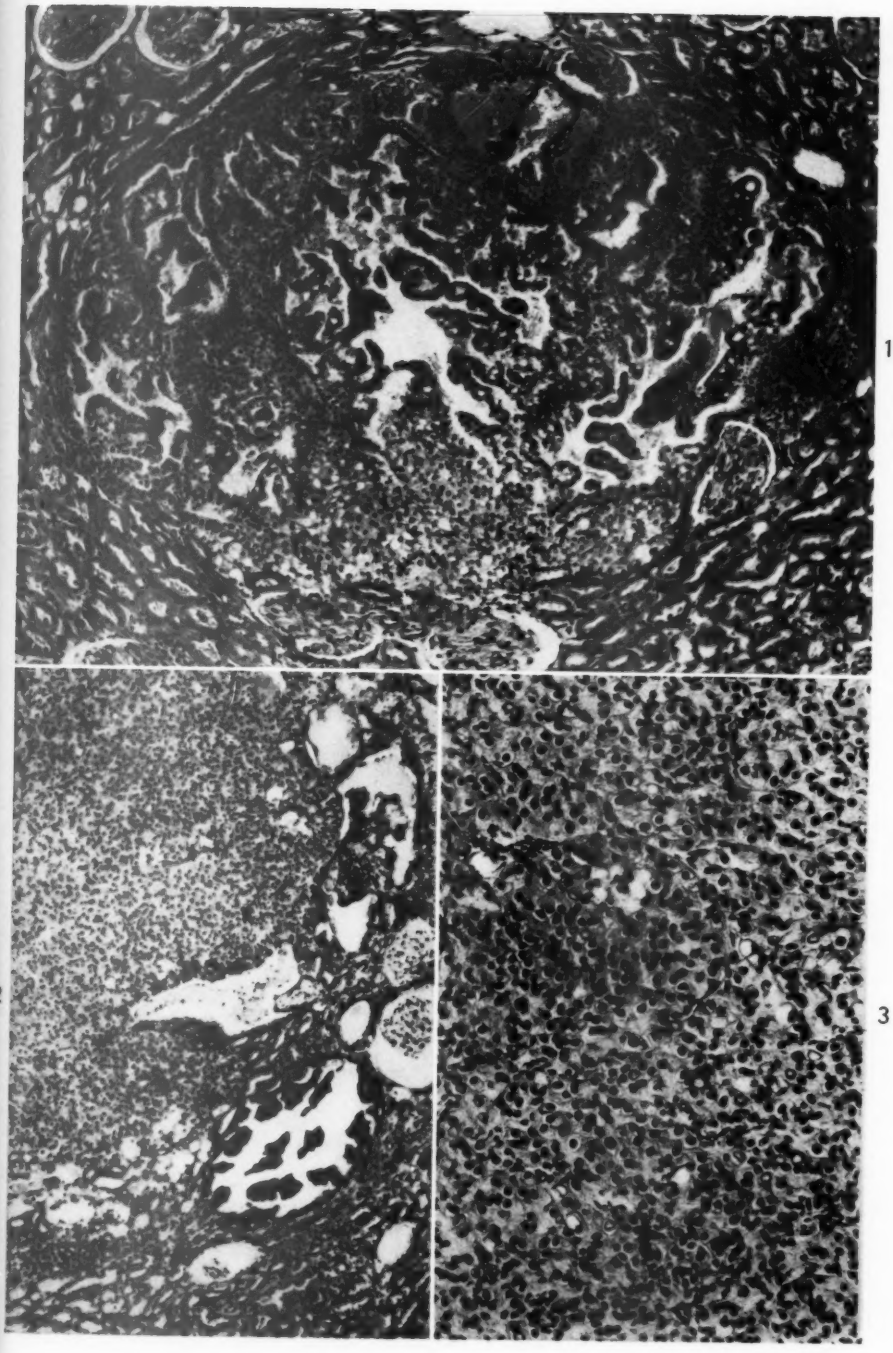
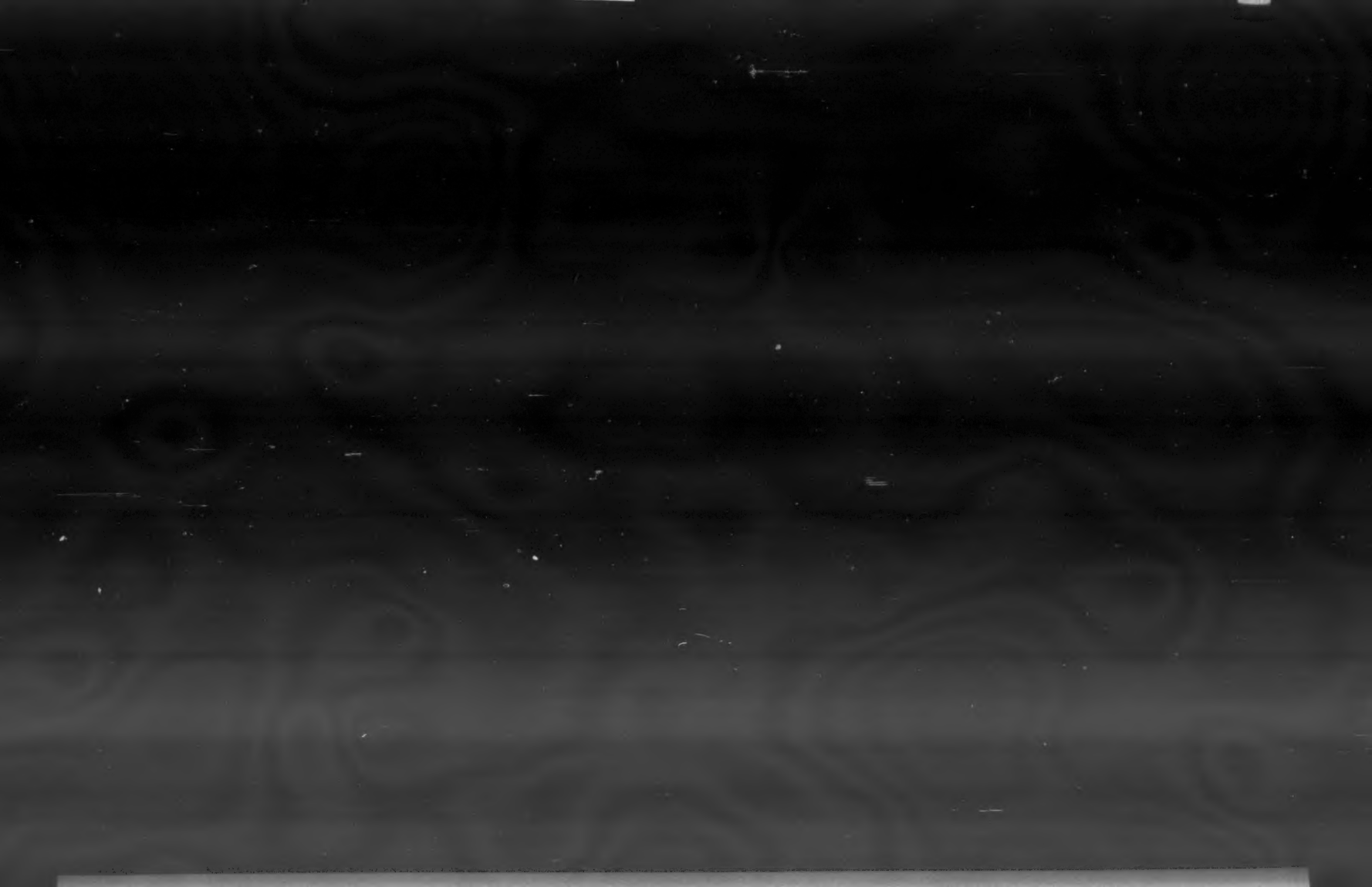


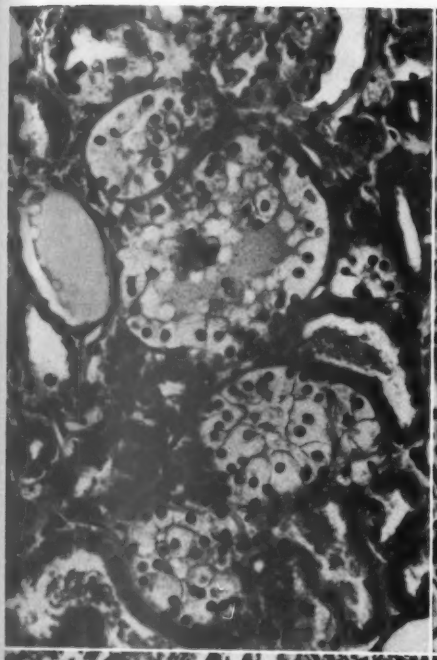
FIG. 4. A proximal convoluted tubule, showing adenomatous proliferation of clear cells. This lesion was in a kidney which contained a large clear cell carcinoma at the opposite pole. Note the focal tubular atrophy and protein casts. × 275.

FIG. 5. A dilated tubule showing early papillary proliferation of the lining cells. There was an eosinophilic adenoma near this lesion. × 275.

FIG. 6. A portion of a large cortical carcinoma. Most of the neoplasm is composed of closely packed clear cells; a papillary lesion is manifest at the left side of the photograph. This tumor ruptured into the retroperitoneal region and caused massive hemorrhage. × 70.

FIG. 7. An undifferentiated malignant tumor contains numerous clefts and pseudoglomerular structures. There is diffuse infiltration of the surrounding parenchyma. × 130.





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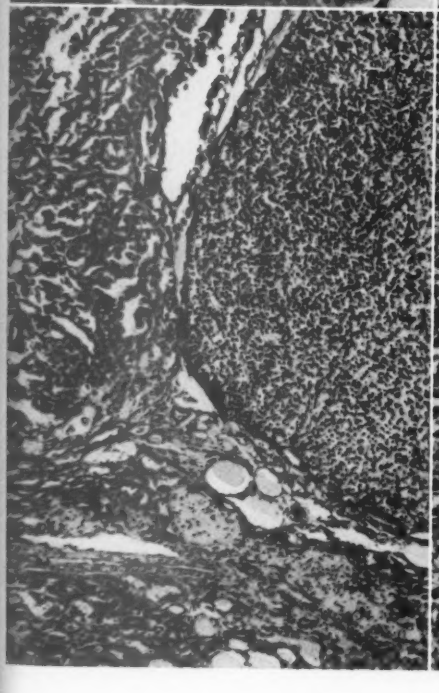

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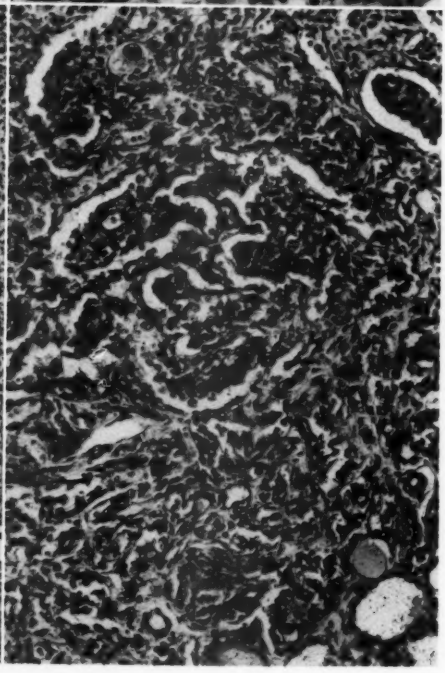

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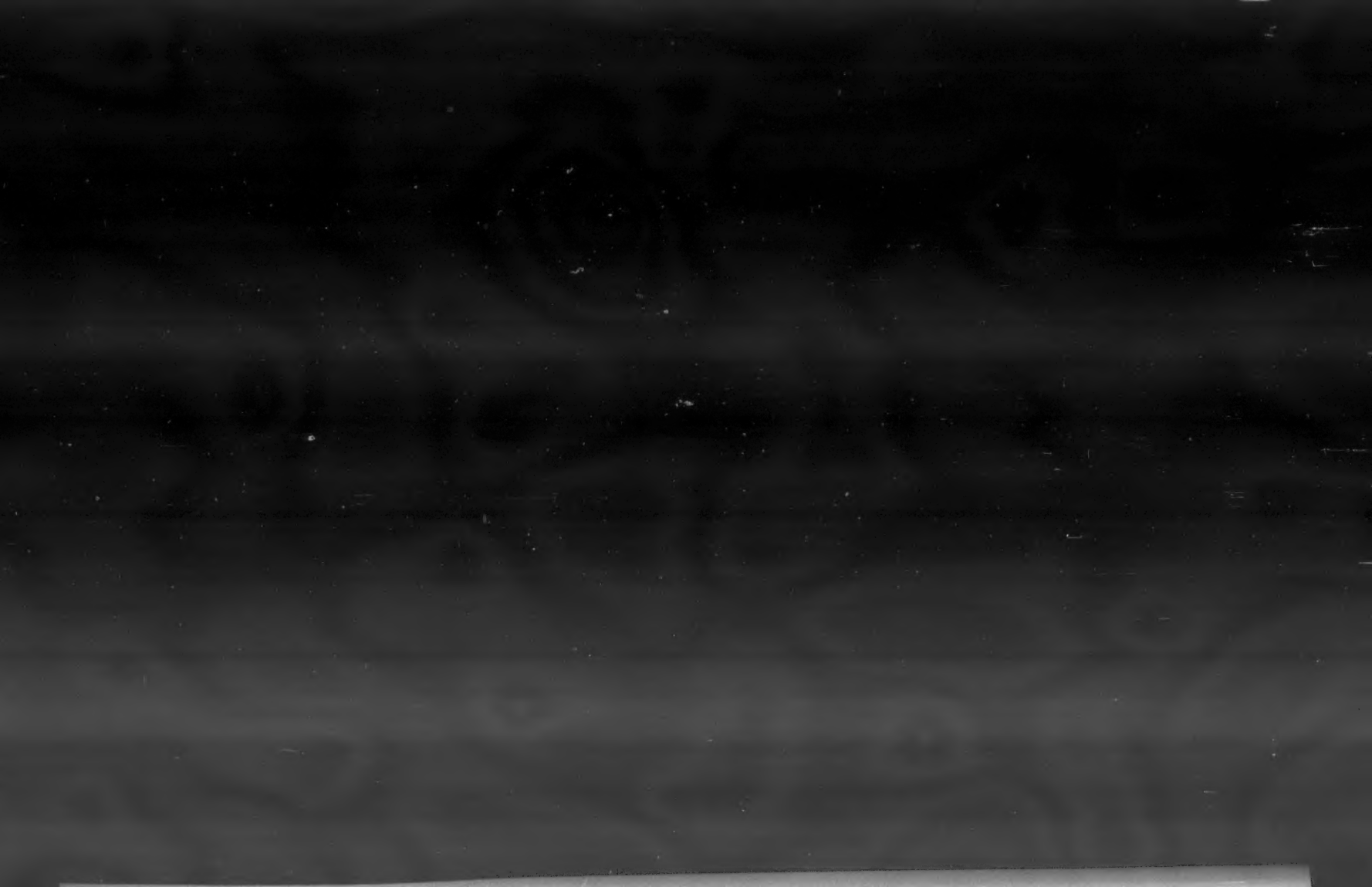
FIG. 8. Clear cell carcinoma. Papillary excrescences and foci of necrosis are shown. × 130.

FIG. 9. Renal cortical carcinoma. The lesion is composed of clear, pleomorphic cell cords. × 130.

FIG. 10. Transitional cell carcinoma. There is diffuse infiltration of the renal parenchyma by well differentiated tumor cells. Extensive renal alteration is attributable to obstruction. × 70.

FIG. 11. Transitional cell carcinoma. There is extensive suppuration in the renal pelvis. Early renal parenchymal invasion is evident. × 120.



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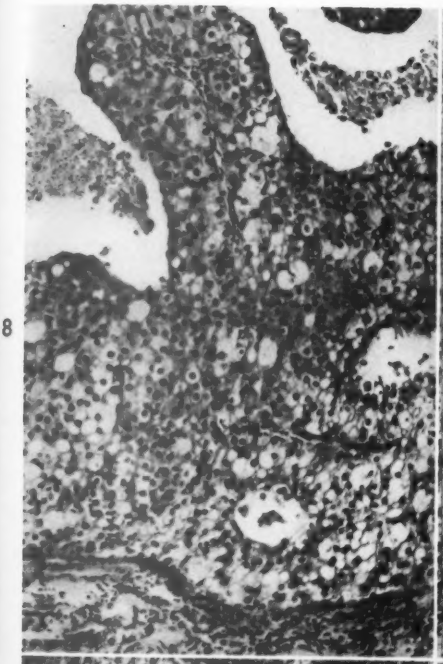

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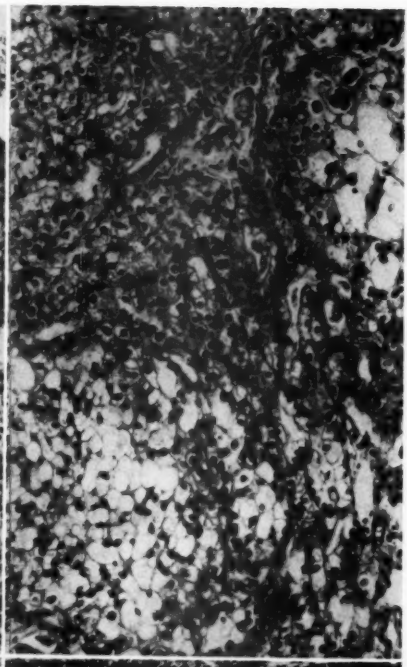

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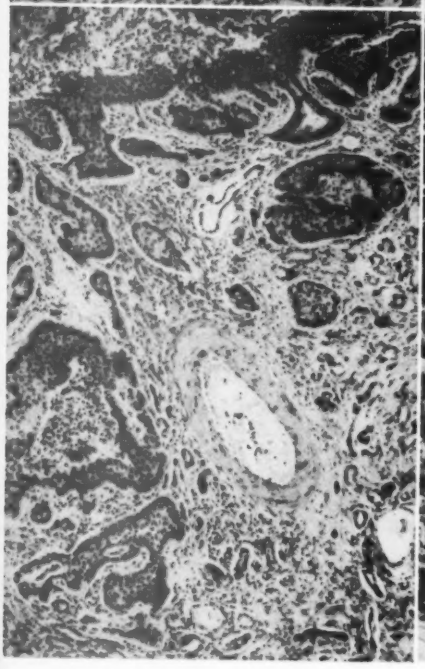

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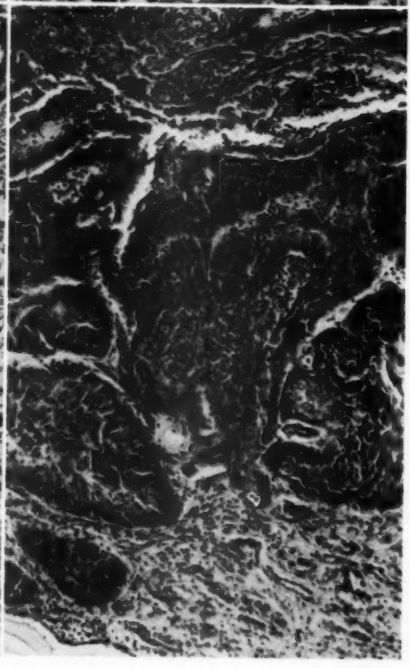

11

CYTOLOGIC STUDIES OF CULTURED CELLS OF THE CLOUDMAN S-91 MOUSE MELANOMA

R. Barishak, M.D.,* S. R. Wellings, M.D.,† and Benjamin V. Siegel, Ph.D.

From the Francis I. Proctor Foundation for Research in Ophthalmology and the Department of Pathology, University of California School of Medicine, San Francisco, Calif.

A transplantable melanoma from mice was reported by Harding and Passey [1] in 1930. In 1937 Cloudman [2] discovered a more malignant type which metastasized more readily and killed the host sooner. An amelanotic form of the Cloudman tumor was obtained subsequently.[3]

Mouse melanomas were investigated in tissue culture by various workers [4–6] who noted that the outgrowths contained macrophages, fibroblasts and melanocytes. Biopsy specimens of the Cloudman S-91 mouse melanoma revealed two histologic patterns: a carcinoma-like structure composed mainly of round or polygonal cells, and a sarcoma-like form composed of spindle cells.[3] Electron microscopy of the neoplasm suggested that the melanin granules formed in the Golgi apparatus [7]; similar evidence has been obtained in the case of human malignant melanoma.[8] By light and electron microscopy, round and spindle-shaped melanocytes, macrophages and fibroblasts were found in outgrowths of human malignant melanoma cultured *in vitro*.[9]

The present study is concerned with the light and electron microscopic appearance of the Cloudman S-91 mouse melanoma cultured *in vitro*.

Material and Methods

Six mice of the DBA strain, bearing subcutaneous transplants of the Cloudman S-91 mouse melanoma, were obtained from the Cancer Research Genetics Laboratory of the University of California in Berkeley. Following sacrifice by cervical fracture, the melanoma tissue was removed under sterile conditions, cut into 1 mm. fragments and explanted in chicken plasma clots on cover slips in Leighton tubes. Tissue fragments were also explanted in roller tubes. The nutrient medium, consisting of 5 per cent chick embryo extract, 20 per cent lamb's serum and 75 per cent Hanks's balanced salt solution, was changed every 3 to 4 days. For light microscopy, cover slips from the Leighton tubes were fixed in absolute methanol and stained with the May-Grünwald-Giemsa stain,[10] or fixed in formol-bromide prior to applying Wilder's reticulum stain.[11a] This last is a silver impregnation method believed to blacken previously colorless premelanin granules in melanocytes.[12,13] Other ex-

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* Research Fellow, National Council to Combat Blindness, Inc. Present address: University of Istanbul, Ziya Gün Institute, Istanbul, Turkey.

† Senior Research Fellow, United States Public Health Service.

plants and outgrowths on cover slips were incubated in tyrosine solution to detect the enzyme tyrosinase.[14] The tyrosine-tyrosinase reaction resulted in the appearance of dark pigment granules in the cytoplasm of melanocytes and was of assistance in the recognition of these cells. The tyrosine-tyrosinase preparations were subsequently fixed in Carnoy's fluid and counterstained by the van Gieson method.[11b]

For electron microscopy, outgrowths were removed from the roller or Leighton tubes with a wire loop and fixed for 2 hours in 1 per cent osmium tetroxide buffered to pH 7.4 with added sucrose.[15] Alternatively, some cultures were fixed in osmic vapor *in situ* for 10 minutes prior to transfer to the osmium tetroxide solution. The fragments of outgrowth were then dehydrated in a graded series of ethyl alcohols and infiltrated with n-butyl methacrylate; polymerization was accomplished at 45° C. for 24 hours. Thin sections were cut with a Porter-Blum microtome equipped with a plate glass knife. These were mounted on carbon-coated copper specimen grids and stained with uranyl acetate.[16] All sections were examined with an RCA EMU 2B electron microscope.

Results

Light Microscopy

The different cell types in the outgrowths from explants of Cloudman S-91 mouse melanoma were, in general, similar to those described in cultures of the Harding-Passey melanoma.[4–6] The cell types recognized were macrophages, fibroblasts and melanocytes.

The macrophages migrated toward the periphery of the clot during the first 24 hours of culture and appeared as heavily pigmented round cells which retained their structure throughout the experiment (Figs. 1 and 2). The granules in the macrophages were dark brown, unequal in size, and usually irregular in shape as compared to those of the melanocytes. Large empty vacuoles were frequently observed in the cytoplasm and were believed to represent spaces occupied by fat droplets previously removed by the action of lipid solvents employed in the preparation of the specimens. The cytoplasm of macrophages was relatively abundant in comparison to the size of the nucleus. Since these cells already possessed brown pigment granules, the silver impregnation method and the tyrosine-tyrosinase reaction were not helpful in establishing their identity.

Fibroblasts were usually less numerous than those observed in cultures of human melanomas.[9] They were large, stellate in shape, and usually possessed several broad cytoplasmic processes. The latter tapered gradually, unlike the processes in melanocytes. Nuclei were usually large and pale; the nuclear-cytoplasmic ratio was relatively small, as in the case of the macrophages. The cytoplasm of the fibroblasts exhibited no dark granules following the tyrosine-tyrosinase reaction or after application of Wilder's reticulum stain. Some cells, adjudged to be fibroblasts by other criteria, contained one or two dark granules similar to those seen in the macrophages.

Melanocytes (Figs. 1 and 2) appeared in the outgrowths on the third or fourth day of culture. They were usually spindle-shaped and were frequently oriented parallel to one another (Figs. 1 and 3). Their nuclei were located at the widest part of the cells, and the cytoplasm projected from both poles as two long, thin processes. These processes resembled threads and were quite unlike the broad, gradually tapering processes of fibroblasts. Brown melanin granules appeared in the cytoplasm of melanocytes after one or more weeks in culture and were smaller and more uniform in size than the granules in macrophages. In addition to the spindle-shaped forms, tripolar melanocytes with 3 threadlike processes were sometimes observed in the outgrowths (Fig. 4). Dark granules appeared in the cytoplasm of previously nonpigmented spindle-shaped and tripolar melanocytes following application of the Wilder reticulum stain or the tyrosine-tyrosinase reaction (Fig. 5).

Electron Microscopy

Melanocytes and macrophages in the outgrowths were examined by electron microscopy. Identification of cell types was based partly on size and shape as established by light microscopy and on certain fine structural features previously described in human malignant melanoma.[9,17]

Spindle-shaped melanocytes were observed most frequently (Figs. 6 and 7). Their cell and nuclear membranes were distinct, and the nuclei were relatively long, narrow and of uniform width; nucleoli were occasionally large. The cytoplasm contained variable numbers of melanin granules which tended to be uniform in size, varying between 100 and 500 μ. The melanin granules were usually composed of smaller granular or rod-shaped internal units (Fig. 9). Mitochondria were rod-shaped or ovoid, limited by the usual double membranes, and contained variable numbers of internal cristas. No transitional forms between mitochondria and melanin granules were observed. Ergastoplasm was sparse, consisting of a variable number of small circular or flattened sacs. "Ribonucleoprotein particles," similar to those described by Palade,[18] were attached to the outer surfaces of the ergastoplasmic membranes and were also scattered singly or in small groups throughout the cytoplasmic matrix. The Golgi substance was generally less abundant than in the instance of human malignant melanocytes cultured *in vitro*.[9] The Golgi apparatus consisted of the usual smooth-surfaced, flattened sacs, often oriented parallel to one another and surrounded by small vesicles (Fig. 11). As in previous studies, the smallest granules were observed in the Golgi region, suggesting the formation of melanin granules in this organelle.[7-9]

The macrophages were larger than the melanocytes, and their cyto-

plasm appeared relatively more abundant in comparison to the size of their nuclei (Fig. 8). Nuclei were ovoid and the chromatin was finely granular; nucleoli were sometimes large. The cytoplasm contained numerous fat droplets, as well as many dense granules which varied in size from 200 to 3,500 μ. These granules also varied in internal structure; some were homogeneously granular while others appeared to be aggregates of melanin granules similar to those seen in the melanocytes. Membranes, fine granules, and other unidentifiable material were frequently seen in the phagocytosed granules (Figs. 8 and 10).

Discussion

Cell types observed in the outgrowths were generally similar to those noted by other workers in tissue cultures of mouse melanomas. The spindle-shaped cells also resembled closely those observed in tissue cultures of human malignant melanomas from skin [9] and uvea.[19] An impressive fact is the similarity of melanoma cells derived from different sources.

The tyrosine-tyrosinase incubation caused dark granules to appear in melanocytes which had no visible melanin granules prior to staining.[14] Wilder's reticulum stain, a silver impregnation method, was believed to blacken colorless premelanin granules already present in the nonpigmented melanocytes.[12,13]

Spindle-shaped bipolar cells with two threadlike terminal processes, one at either end, and tripolar cells with 3 similar processes were seen in the outgrowths by light microscopy. When these cells were pigmented, their cytoplasm contained small, uniform, dark brown or black granules. Similar granules almost invariably appeared in nonpigmented cells of identical structure after the application of the tyrosine-tyrosinase and silver impregnation methods. These results would indicate that the spindle-shaped bipolar cells and the tripolar cells were melanocytes.

On the other hand, the larger typical fibroblasts possessed 3 or more broad, cytoplasmic processes which tapered gradually. These cells never contained dark pigment granules of uniform size similar to those seen in the melanocytes, and they never acquired such uniform granular pigmentation following the use of tyrosine-tyrosinase and the silver impregnation methods.

Fibroblasts were considerably less numerous than in tissue cultures of human malignant melanoma.[9,19] In this connection, Algire, Loustalot, Legallais and Anderson [3] observed that the Cloudman S-91 melanoma in histologic sections contained only a small amount of connective tissue.

Portions of the outgrowths rich in spindle-shaped melanocytes and macrophages were selected for electron microscopy. For this purpose,

morphologic criteria established by light microscopy with the aid of the special stains were useful in selecting suitable parts of the outgrowths. The electron microscopic appearance of the melanocytes and macrophages corresponded closely to that previously described in human malignant melanoma cells cultured *in vitro*.[9]

By electron microscopy, spindle-shaped melanocytes were readily found in the outgrowths. These cells contained relatively small melanin granules of uniform size, composed of smaller granular or rod-shaped internal units. Objects resembling small melanin granules were observed in the Golgi regions of the melanocytes. Similar morphologic evidence suggested the formation of melanin granules in the Golgi apparatus of biopsy tissue from both the Cloudman S-91 mouse melanoma[7] and human malignant melanoma,[8] as well as of tissue cultures of human malignant melanoma.[9]

The electron microscopic pattern of cells interpreted as macrophages also closely resembled that previously described in human malignant melanoma cells *in vivo* and *in vitro*.[9,17] The granules of the macrophages were larger and more irregular in size than those in melanocytes. Many of the granules in the macrophages appeared to be composed of aggregates of numerous smaller melanin granules similar to those observed in melanocytes. The phagocytosed granules also frequently contained other membranous and granular debris of uncertain origin. These observations suggested that the granules observed in the macrophages were the result of the phagocytosis of relatively large masses of melanocytic cytoplasm by the macrophages. The presence of numerous cytoplasmic fat droplets in macrophages was in agreement with light microscopic observations of empty cytoplasmic vacuoles after extraction by lipid solvents.

Summary

Cloudman S-91 mouse melanoma was cultured *in vitro* by the explant method. The cells in the outgrowth were examined by light and electron microscopy. Spindle-shaped bipolar melanocytes, tripolar melanocytes, macrophages and fibroblasts were observed, and the tyrosine-tyrosinase reaction and silver impregnation method were utilized in the identification of melanocytes. Electron microscopy provided further evidence for the formation of melanin granules in the Golgi region. Melanin granules of melanocytes were relatively uniform in size and composed of granular and rod-shaped subunits. The granules of macrophages were larger and more variable in size and often contained several melanin granules similar to those seen in the melanocytes.

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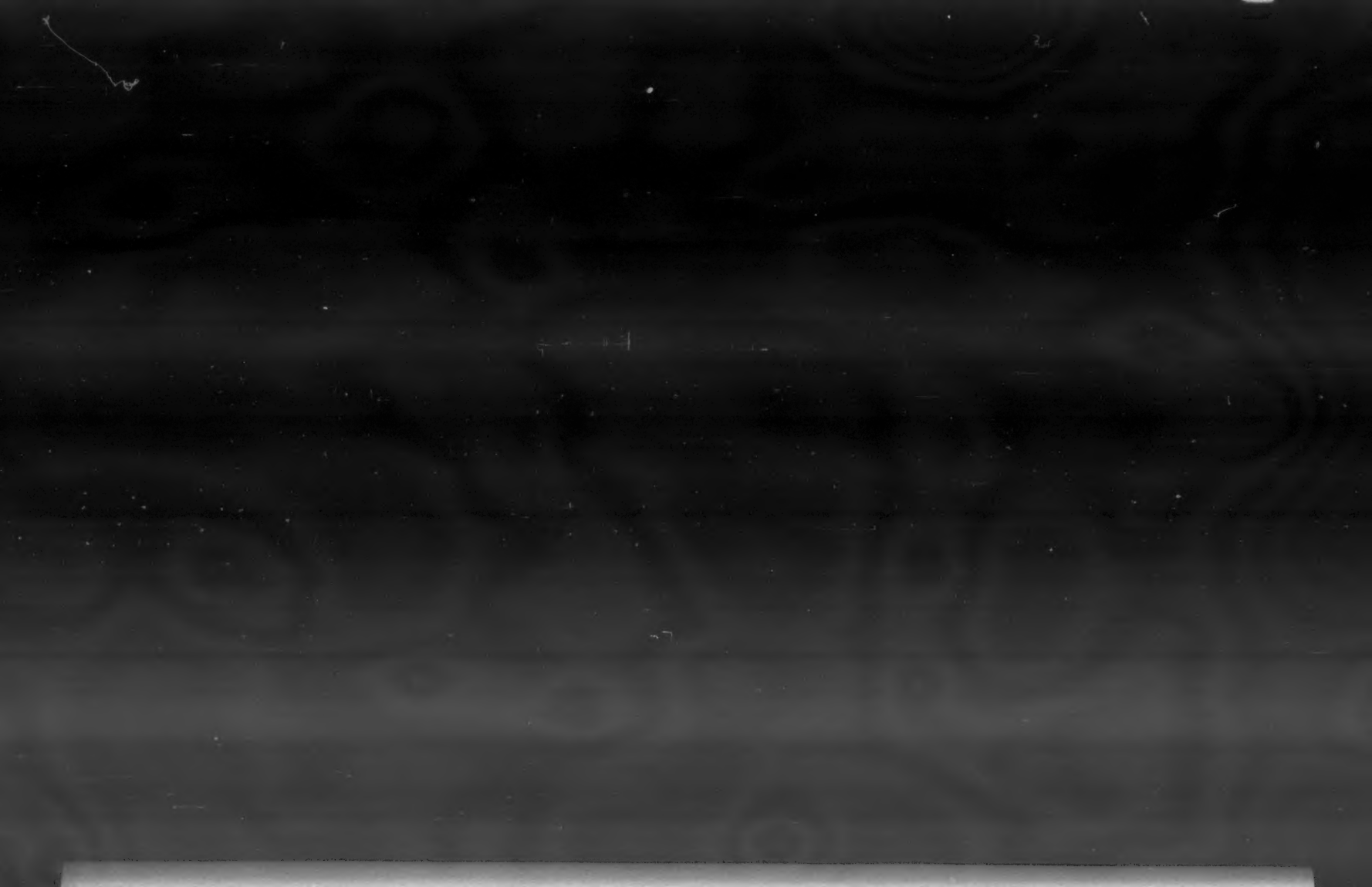
[*Illustrations follow*]

Legends for Figures

Fig. 1. A central group of spindle-shaped melanocytes (ME) contain a few small, uniform melanin granules. Numerous round macrophages (MA) contain dark granules of variable size and empty-appearing vacuoles. Thirteen days in culture; Wilder's reticulum stain. × 300.

Fig. 2. Melanocytes (ME) and macrophages (MA) in outgrowth 13 days in culture. Wilder's reticulum stain. × 300.

Fig. 3. Spindle-shaped melanocytes are oriented parallel to one another in a 15-day tissue culture. May-Grünwald Giemsa stain. × 330.



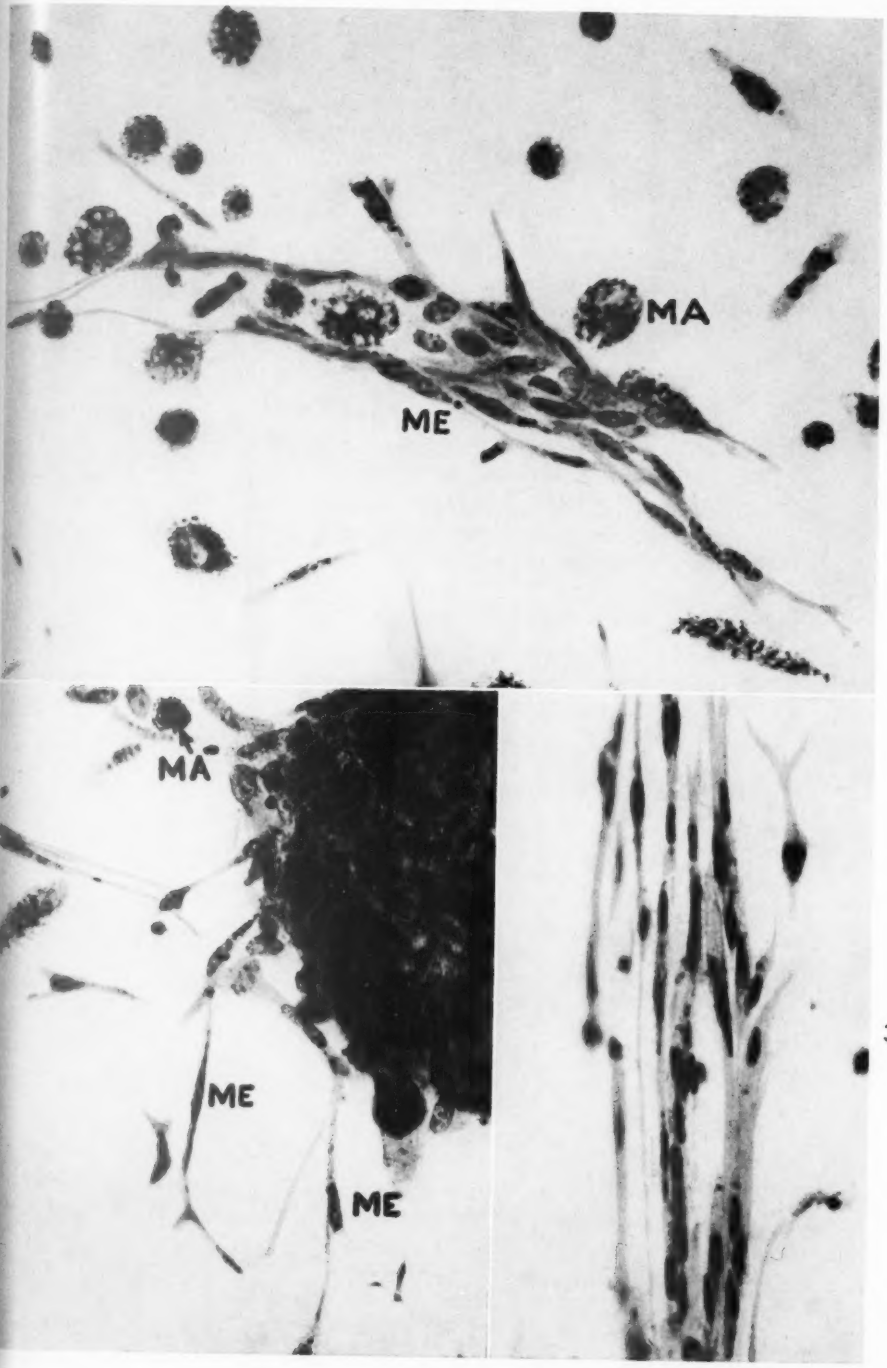

MA
ME
1
MA
ME
ME
3

FIG. 4. A group of melanocytes, many of which are tripolar, in a 33-day tissue culture. May-Grünwald-Giemsa stain. × 135.

FIG. 5. A spindle-shaped melanoma cell with pigment granules (arrow) which appeared as a result of tyrosine-tyrosinase reaction; 13 days in culture. Tyrosine-tyrosinase reaction counterstained with van Gieson stain. × 345.

Figures 6 to 10 are electron micrographs.

FIG. 6. Parts of two spindle-shaped melanocytes, showing cell membrane (CM), nuclei (N), mitochondria (M) and melanin granules (ME). Remnants of necrotic cells are present in the lower right of the illustration. × 5700.

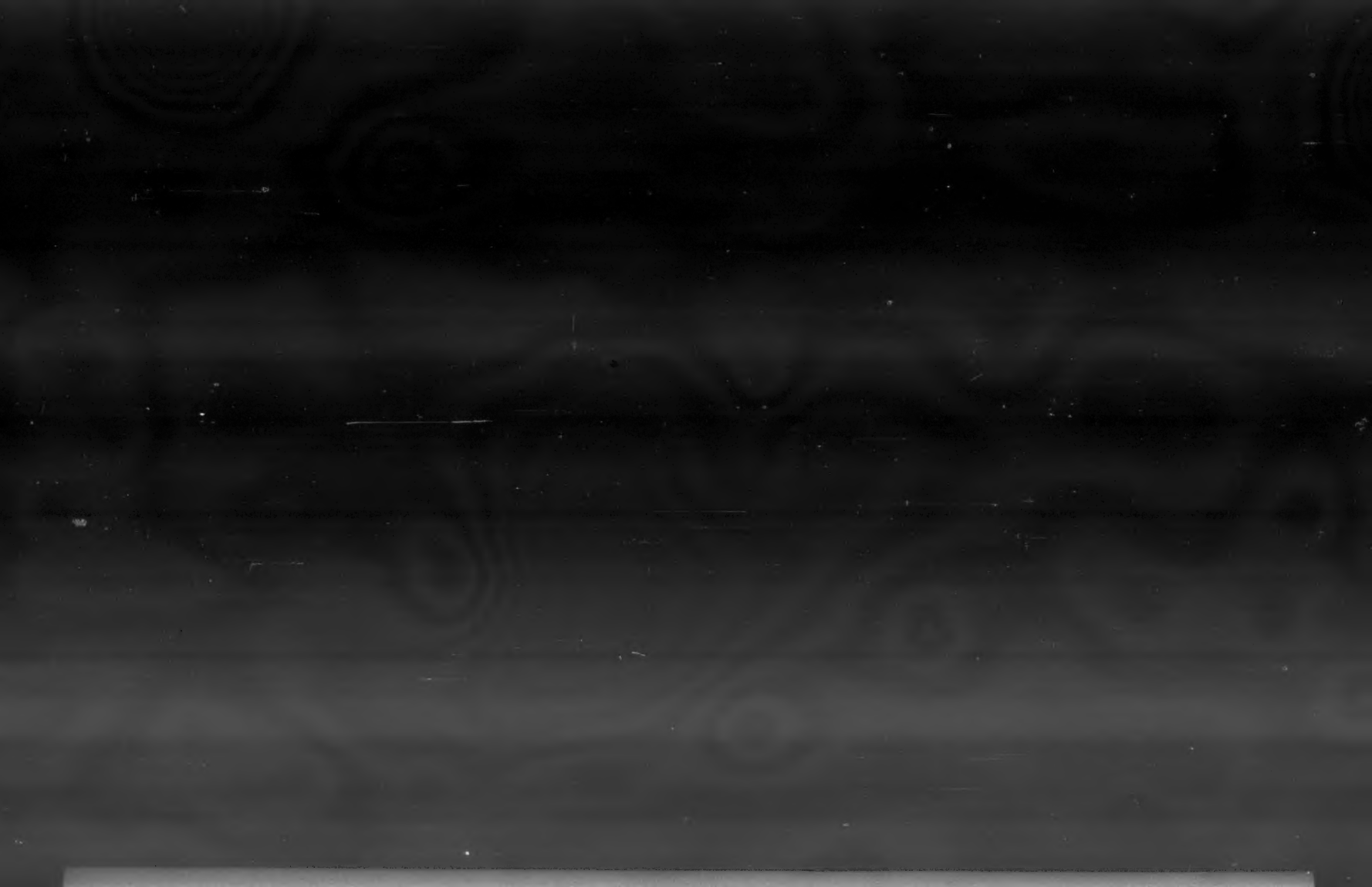


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FIG. 7. Spindle-shaped melanocyte, showing nucleus (N), cell membrane (CM), melanin granules (ME) and mitochondria (M). × 7000.

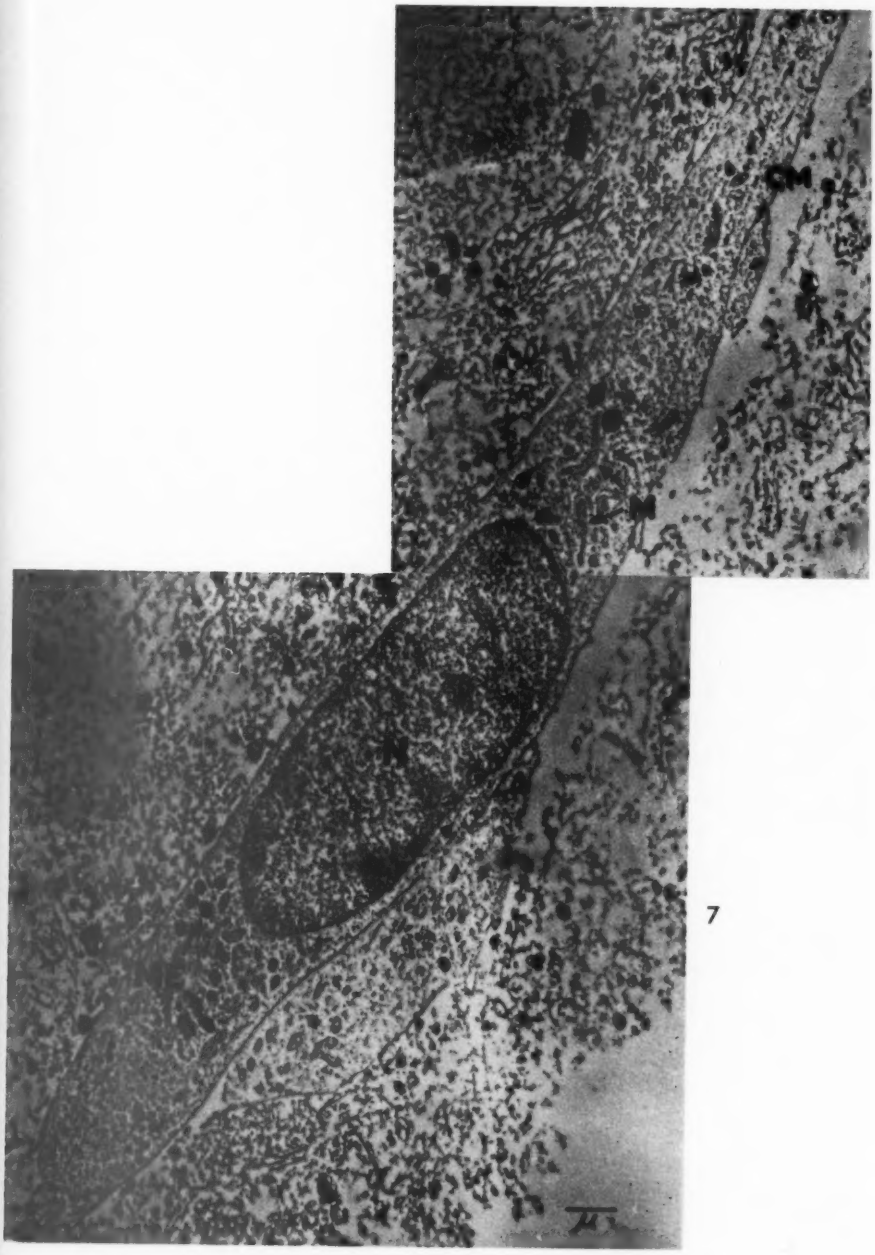

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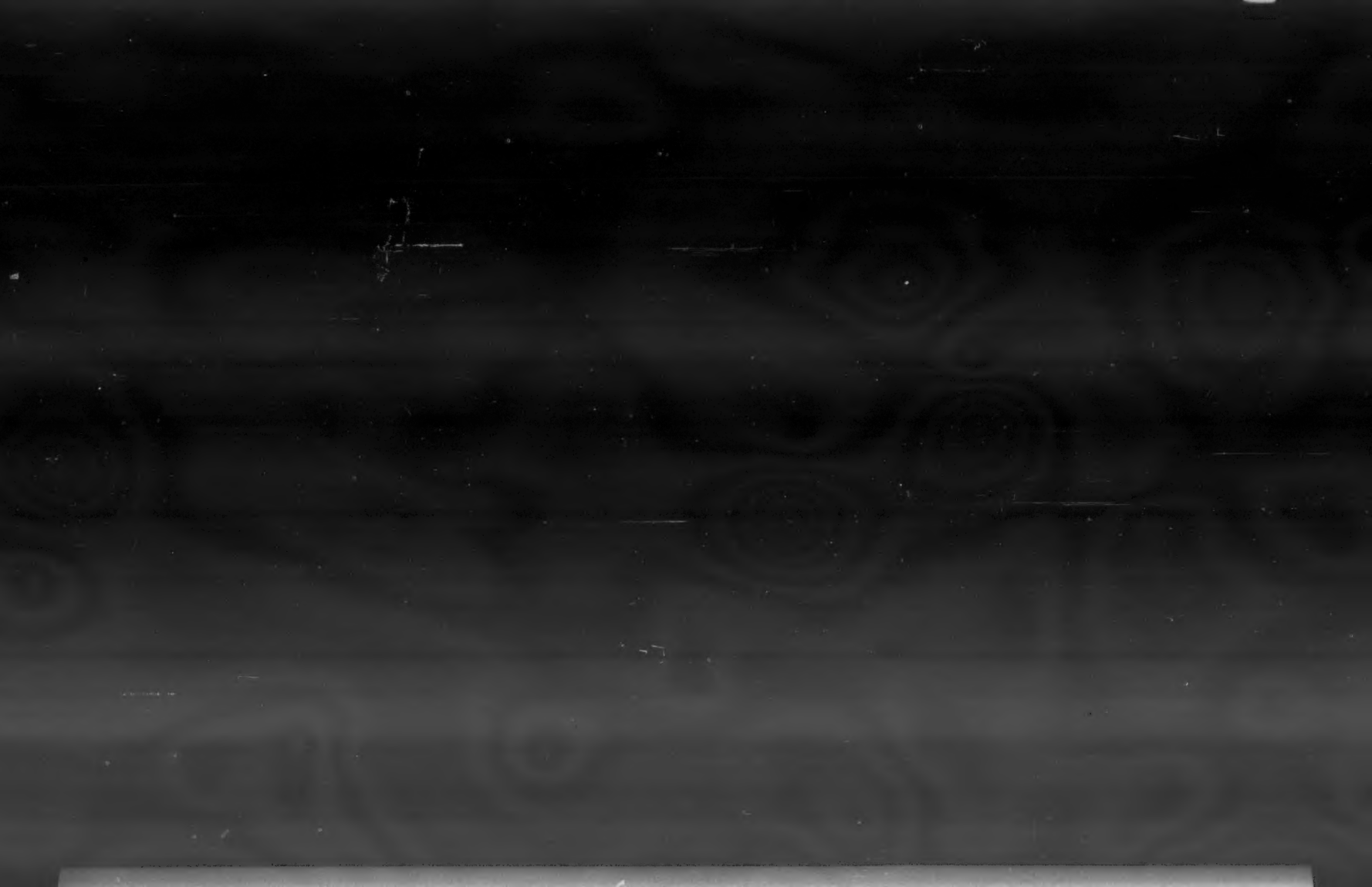
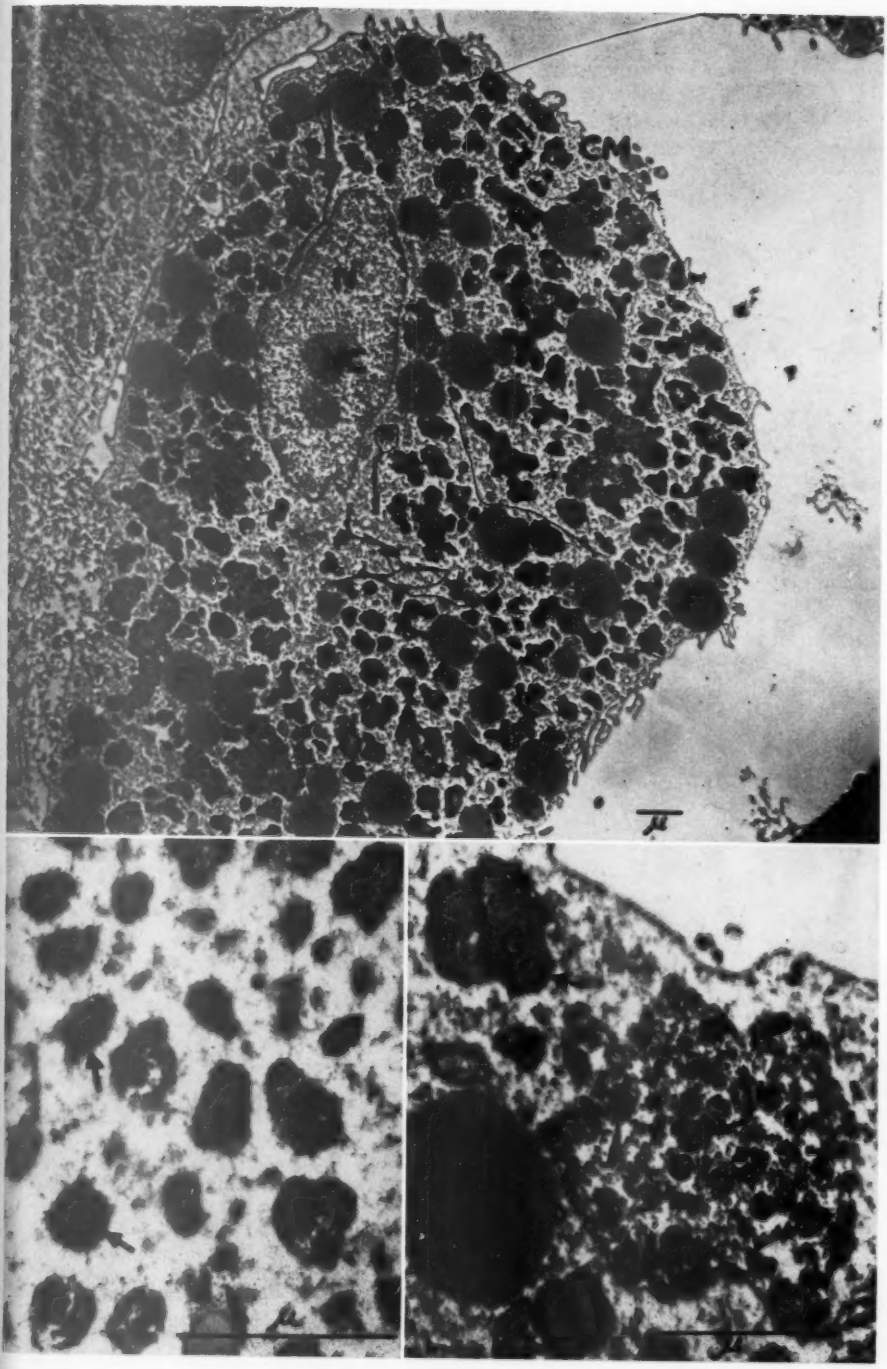
FIG. 8. Macrophages with a nucleus (N), nucleolus (NC), cell membrane (CM), fat droplets (F) and dense granules of variable size (G). × 6200.

FIG. 9. Part of a melanocyte showing typical melanin granules composed of smaller granular and rod-shaped subunits (arrows). × 33,000.

FIG. 10. Part of a macrophage, illustrating the characteristic conglomerate granules (G) composed of melanin granule aggregates in a matrix of finely granular material. The upper granule has a dense, finely granular matrix, while the lower has a more loosely aggregated granular matrix surrounding the constituent melanin particles. Rod-shaped subunits of the melanin granules are similar to those in Figure 9 (arrows). A fat droplet is shown (F). × 26,000.





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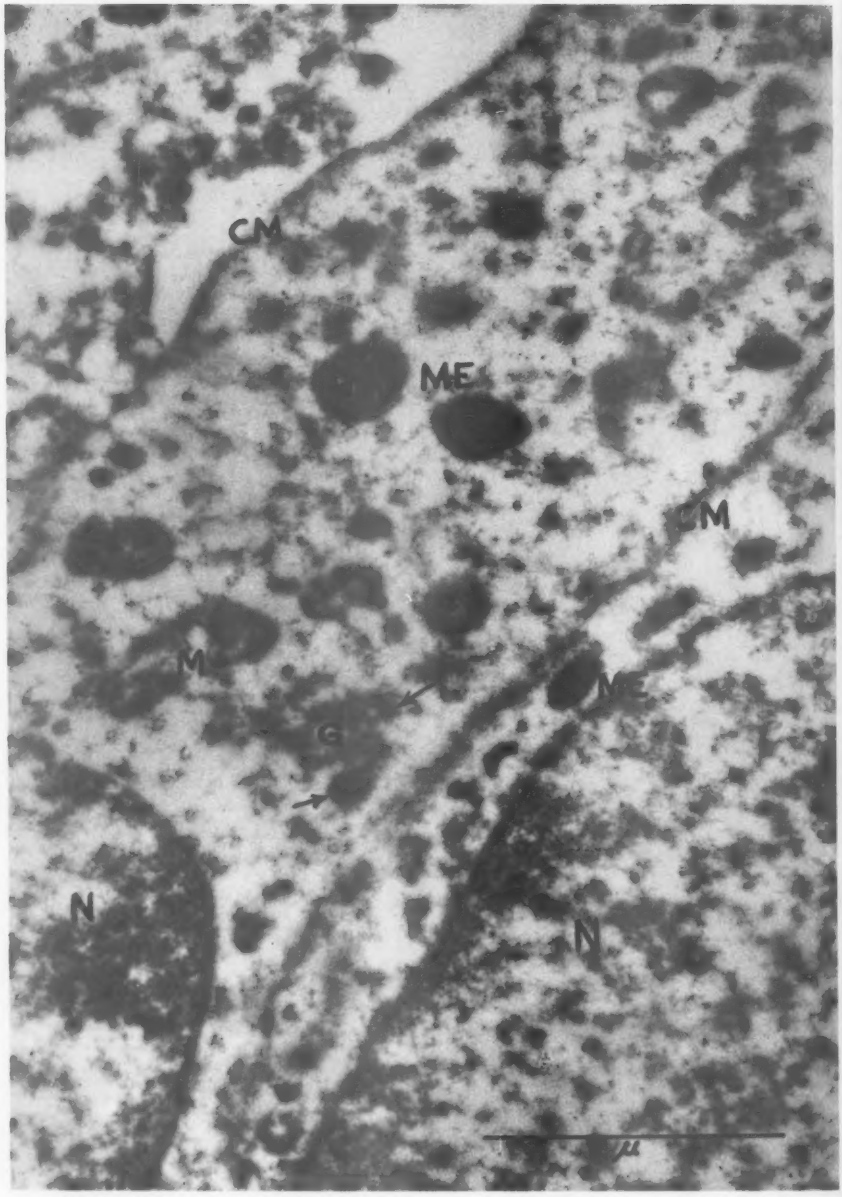

FIG. 11. Portions of two melanocytes. A small group of vesicles and tubules of the Golgi apparatus (G) is associated with two small melanin granules (arrows). Melanin granules (ME), mitochondria (M), nuclei (N) and cell membranes (CM). × 44,000.

THE EFFECT OF SERUMS FROM TUMOR SUSCEPTIBLE AND NONSUSCEPTIBLE GUINEA PIGS ON LYMPHOSARCOMA 6C3HED IN C3H MICE

PETER A. HERBUT, M.D.; WILLIAM H. KRAEMER, M.D.; FRANCIS A. MCKEON, M.D., AND RICHARD C. TAYLOR, M.D.

From the Elizabeth Storck Kraemer Memorial Foundation and Department of Pathology, Jefferson Medical College and Hospital, Philadelphia, Pa.

Spontaneous tumors in the guinea pig are rare. In a thorough review of the world's literature, Rogers and Blumenthal [1] were able to collect only 124 recorded cases. To these they added 14. All of the latter occurred in 4,000 guinea pigs of an inbred susceptible strain, and all developed in animals over 3 years of age. Since less than 2.5 per cent of the guinea pigs in this strain lived over 3 years, the incidence of spontaneous tumors in animals that lived longer than 3 years was 14.4 per cent. In contrast, the incidence of spontaneous tumors in 2,000 animals of an inbred nonsusceptible strain was zero. The number of pigs in this group that lived over 3 years of age was, however, only one third that of the susceptible strain. Of the total of 138 tumors recorded, only 49 were considered malignant, and none of these, with the exception of 10 examples of leukemia, showed metastasis.

Why the guinea pig is less susceptible to neoplasia than most other laboratory animals is not known. It is established, however, that the guinea pig is unique in that it normally possesses a powerful tumor inhibitory principle (TIP) in its serum. This is effective against transplantable lymphosarcoma 6C3HED in C3H mice, lymphoma II in strain A mice, and Murphy-Sturm lymphosarcoma in Wistar rats.[2-5] The question naturally arises as to whether TIP is responsible for the innate protection of this species against the development of tumors. If this is true, it is possible that serum from Rogers' susceptible strain of guinea pigs might contain less TIP than the serum in the nonsusceptible strain. Accordingly, we thought it might be of interest to compare the tumor inhibitory activity of serum from each of Rogers' strains of guinea pigs with that of serum from guinea pigs generally used in our own laboratory.

MATERIAL AND METHODS

The test tumor was the Gardner lymphosarcoma 6C3HED carried subcutaneously by C3H mice. The latter, weighing approximately 20 gm. each, were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The donor tumor was 9 days old. It was implanted into the subcutaneous tissues of the right flank

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by the usual trocar technique. Treatment was started on the fourth day after implantation, at which time the tumors measured approximately 9 mm. in diameter. The donor guinea pigs were of 3 groups: (a) stock, of no particular strain, approximately 1 year of age, purchased from Research Supply Company, Philadelphia, and used for normal guinea pig serum; (b) Rogers' susceptible strain, one group 4 years of age or older and a second group approximately 1 year of age, supplied by Dr. James B. Rogers, and used for susceptible guinea pig serum; and (c) Rogers' nonsusceptible strain, one group 4 years of age or older and a second group approximately 1 year of age, also supplied by Dr. Rogers, and used for nonsusceptible guinea pig serum.

All serums were prepared in our own laboratory. The guinea pigs were narcotized with pentobarbital sodium and bled from the heart. The serum was separated off by centrifugation and sterilized by Seitz filtration. It was formulated for use by diluting 1 part of serum with 2 parts of physiologic saline.

The testing was carried out in 2 separate experiments, one using test serum from 4-year-old guinea pigs and the other using test serum from 1-year-old guinea pigs (Tables I and II). In each, the control serum was from 1-year-old guinea pigs because 4-year-old animals were not available. All injections were given intravenously on the fourth, fifth and sixth days after implantation at dose levels of 1.2 ml., 1 ml., 0.8 ml., 0.6 ml., 0.4 ml., and 0.2 ml. The experiments were terminated on the eighth day after implantation. The tumors were measured (average of 3 dimensions) and the animals were weighed on the fourth, sixth and eighth days after implantation. Necropsies were performed on all animals, and histologic examination was carried out on the liver, kidney, and tumor or tumor site sporadically. The criteria for complete regression of tumor was its gross disappearance while that for favorable response was an appreciable decrease in size of the tumor (generally more than 2 mm. in average diameter) as compared with nontreated control animals.

Results

The results of both experiments are tabulated in Tables I and II. In the first experiment, the tumor inhibitory activity of normal guinea pig serum was distinctly greater than that of either susceptible or nonsusceptible 4-year guinea pig serum. This is indicated by (a) complete regression of tumor in 10 of 10 mice treated with 1.2 ml. and 9 of 10 mice treated with 1 ml. of normal guinea pig serum with no complete regression of tumor in either of the other 2 groups; and (b) consistent (although slight) decrease in sizes of tumors in mice treated with normal guinea pig serum as compared with sizes of tumors in mice treated with corresponding amounts of the other 2 serums. In the latter, the tumor inhibitory response of serum from susceptible 4-year-old guinea pigs was slightly, but consistently, just a little better than that of serum from nonsusceptible 4-year-old guinea pigs. In the second experiment, the tumor inhibitory activity of each of the 3 serums, namely, normal 1 year, susceptible 1 year, and nonsusceptible 1 year, was approximately the same.

Discussion

There is no doubt, as indicated above, that the tumor inhibitory activity of serum from normal 1-year-old guinea pigs was greater than that

TABLE I

THE EFFECT OF GUINEA PIG SERUMS ON LYMPHOSARCOMA 6C3HED IN C3H MICE

Treatment	No. mice used	No. of mice surviving 8 days	No. of growths 8 days	Diam. tumors (mm.) Days after implant. 4	6	8	Wt. of mice (gm.) Initial	8 days
None	20	20	20	8.8	14.9	18.9	23.0	29.3
1.2 ml., normal 1 yr.	10	10	0	9.2	6.7	0	22.8	23.3
1.0 ml., normal 1 yr.	10	10	1	8.4	7.2	6.0	23.0	23.3
0.8 ml., normal 1 yr.	10	10	8	9.0	8.4	6.6	23.4	23.7
0.6 ml., normal 1 yr.	10	10	10	8.7	9.3	9.3	21.8	24.5
0.4 ml., normal 1 yr.	10	10	10	8.3	10.6	12.4	23.6	26.1
0.2 ml., normal 1 yr.	10	10	10	8.2	11.6	15.0	22.3	25.5
1.2 ml., susceptible 4 yr.	10	10	10	8.9	8.4	8.4	24.0	24.4
1.0 ml., susceptible 4 yr.	10	10	10	8.3	8.3	9.7	21.8	22.9
0.8 ml., susceptible 4 yr.	10	10	10	8.5	10.2	11.4	23.7	25.6
0.6 ml., susceptible 4 yr.	10	10	10	8.7	10.0	12.1	23.0	25.1
0.4 ml., susceptible 4 yr.	10	10	10	8.8	11.8	14.8	22.8	26.2
0.2 ml., susceptible 4 yr.	10	10	10	8.2	13.2	16.2	21.3	25.7
1.2 ml., nonsusceptible 4 yr.	10	10	10	9.0	9.0	9.3	24.1	24.0
1.0 ml., nonsusceptible 4 yr.	10	10	10	8.4	9.3	10.0	22.8	23.4
0.8 ml., nonsusceptible 4 yr.	10	10	10	8.7	11.0	13.7	22.2	25.9
0.6 ml., nonsusceptible 4 yr.	10	10	10	8.1	11.2	14.1	22.7	28.0
0.4 ml., nonsusceptible 4 yr.	10	10	10	9.1	12.7	16.3	22.1	26.3
0.2 ml., nonsusceptible 4 yr.	10	10	10	9.0	14.0	18.3	23.8	29.2
None	20	20	20	9.2	13.5	19.0	20.4	27.7
1.2 ml., normal 1 yr.	10	10	3	8.4	6.4	8.5	24.3	25.2
1.0 ml., normal 1 yr.	10	10	8	8.9	7.2	7.7	21.8	22.4
0.8 ml., normal 1 yr.	10	10	9	9.2	9.0	10.5	25.8	27.8
0.6 ml., normal 1 yr.	10	10	10	9.0	9.7	12.5	26.5	27.7
0.4 ml., normal 1 yr.	10	10	10	8.9	10.9	14.6	25.0	27.3
0.2 ml., normal 1 yr.	10	10	10	9.1	13.0	18.5	25.9	30.2
1.2 ml., susceptible 1 yr.	10	10	6	9.0	7.4	7.3	24.7	24.9
1.0 ml., susceptible 1 yr.	10	10	10	9.0	7.8	8.3	23.5	25.2
0.8 ml., susceptible 1 yr.	10	10	10	8.9	8.7	9.3	24.0	24.0
0.6 ml., susceptible 1 yr.	10	10	10	9.2	10.7	13.0	23.6	26.6
0.4 ml., susceptible 1 yr.	10	10	10	8.7	11.1	14.1	22.8	25.8
0.2 ml., susceptible 1 yr.	10	10	10	8.9	12.6	17.6	22.9	27.9
1.2 ml., nonsusceptible 1 yr.	10	10	6	8.6	7.3	8.1	23.2	24.4
1.0 ml., nonsusceptible 1 yr.	10	10	10	9.3	9.1	9.1	24.3	25.0
0.8 ml., nonsusceptible 1 yr.	10	10	10	8.7	9.3	10.8	24.2	26.2
0.6 ml., nonsusceptible 1 yr.	10	10	10	8.9	10.0	11.8	23.7	24.8
0.4 ml., nonsusceptible 1 yr.	10	10	10	8.2	10.9	14.5	22.4	26.6
0.2 ml., nonsusceptible 1 yr.	10	10	10	8.3	12.3	17.4	22.5	27.3

All injections given intravenously on the fourth, fifth and sixth days after tumor implantation.
Normal: serum from normal (stock) guinea pigs.
Susceptible: serum from Rogers' susceptible strain of guinea pigs.
Nonsusceptible: serum from Rogers' nonsusceptible strain of guinea pigs.
1 yr.: guinea pigs approximately 1 year old; 4 yr.: guinea pigs over 4 years of age.

of serum from either susceptible or nonsusceptible 4-year-old pigs. While the tumor inhibitory activity of serum from susceptible 4-year-olds was slightly greater than that from the nonsusceptible 4-year-old pigs in the experiment performed, the difference is not significant, for with different groups of animals of the same strains the reverse might just as well have

TABLE II

FINAL DIAMETER (MM.) OF LYMPHOSARCOMA 6C3HED TREATED WITH GUINEA PIG SERUM

Dose	Normal	Susceptible	Nonsusceptible
	1 yr.	4 yr.	4 yr.
1.2 ml.	0	8.4	9.3
1.0 ml.	6.0 (1 tumor)	9.7	10.0
0.8 ml.	6.6 (8 tumors)	11.4	13.7
0.6 ml.	9.3	12.1	14.1
0.4 ml.	12.4	14.8	16.3
0.2 ml.	15.0	16.2	18.3
0 ml.	18.9	18.9	18.9
	1 yr.	1 yr.	1 yr.
1.2 ml.	8.5 (3 tumors)	7.3 (6 tumors)	8.1 (6 tumors)
1.0 ml.	7.7 (8 tumors)	8.3	9.1
0.8 ml.	10.5 (9 tumors)	9.3	10.8
0.6 ml.	12.5	13.0	11.8
0.4 ml.	14.6	14.1	14.5
0.2 ml.	18.5	17.6	17.4
0 ml.	19.0	19.0	19.0

been the case. Such slight variations from one to another batch of guinea pig serums are constantly encountered. This is adequately demonstrated even in the two experiments here recorded. Note, for example, that in the first experiment, serum from normal 1-year-old guinea pigs produced complete regression in 10 of 10 tumors at a dose level of 1.2 ml. and 9 of 10 at a dose level of 1 ml. In the second experiment a different batch of serum from normal 1-year-old guinea pigs produced complete regression in 7 of 10 tumors at a dose level of 1.2 ml. and 2 of 10 at a dose level of 1 ml. This is about as wide a variation in tumor inhibitory activity of normal guinea pig serum as we have encountered. Yet, if the second normal were substituted for the first normal, the serums from susceptible and nonsusceptible 4-year-old pigs would still show less tumor inhibitory activity than the control. Since the tumor inhibitory activity of serum from susceptible and nonsusceptible 1-year-old pigs was as good as that of the corresponding control serum, it is apparent that there is no real demonstrable difference in tumor inhibitory activity of the serum from the strains of guinea pigs tested. The apparent difference demonstrated

in the first experiment can be accounted for by the difference in age of the donor guinea pigs.

Finally, to ascertain any variations in protein and glycoprotein content, each of the 6 groups of serums were analyzed by paper electrophoresis. The patterns were identical.

Summary

Serums from ordinary laboratory guinea pigs, along with those from Rogers' tumor susceptible and tumor nonsusceptible strains of guinea pigs, were tested for tumor inhibitory activity against the Gardner lymphosarcoma 6C3HED carried subcutaneously by C3H mice. Serums from guinea pigs 4 years of age or older caused less retardation of growth than serums from 1-year-old animals. Otherwise, there was no significant difference in tumor inhibitory activity.

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